Immunoassay Kit
Catalog # KCA0021

Canine C-Reactive Protein

BioSource International, Inc.
542 Flynn Road
Camarillo, California 93012 USA
Tel: 805-987-0086 • 800-242-0607
FAX: 805-987-3385
email: tech.support@biosource.com

BioSource Europe S.A.
Rue de l’Industrie, 8
B-1400 Nivelles, Belgium
Tel: +32 67 88 99 99
FAX: +32 67 88 99 96
email: tech.support@biosource.be
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INTENDED USE

The BioSource Canine C-Reactive Protein (CRP) ELISA assay is designed to detect the acute phase protein, C-Reactive Protein, from the serum of dogs. The activation of the body’s immune system-mediated defense mechanisms is termed the acute phase response. Activation can occur due to infection, inflammation, tissue injury, neoplastic growth or immunological disorder.

INTRODUCTION

CRP is one of the family of acute phase proteins found in the blood of both humans and animals\textsuperscript{1,2}. Under normal conditions it is found in low levels in the blood but can increase significantly in response to inflammatory conditions, infections and other disease states where tissue necrosis occurs, and therefore provides a highly sensitive indicator for these conditions.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.
PRINCIPLE OF THE METHOD

The BioSource Canine CRP kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). An antibody specific for Canine CRP has been coated onto the wells of the microtiter strips provided. Samples, including standards of known CRP content, control specimens, and unknowns, are pipetted into these wells.

During the first incubation, the Canine CRP antigen binds to the immobilized (capture) antibody on one site. After washing to remove any unbound material, an HRP labeled anti-canine-CRP antibody specific for canine CRP is added to each well. During the second incubation, this antibody binds the immobilized canine CRP captured during the first incubation.

After removal of excess second antibody, TMB substrate solution is added, which is acted upon by the bound enzyme to produce color. This binds to the HRP labeled antibody to complete the three-member sandwich. The intensity of the color produced is directly proportional to the concentration of CRP present in the original specimen.
REAGENTS PROVIDED

Note: This kit should be stored at 2 to 8°C and used before the expiration date.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>96 Test Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein Standard. Contains 0.01% Thimerosal; 1.5 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>Assay Diluent Concentrate (20x). Contains 0.01% Thimerosal; 50 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>Anti-Canine CRP Conjugate. Contains 0.01% Thimerosal; 11 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>Wash Buffer Concentrate (20x). Contains 0.01% Thimerosal; 50 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>TMB Substrate, ready to use; 11 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>Stop Solution, ready to use; 11 mL per vial.</td>
<td>1 vial</td>
</tr>
<tr>
<td>Canine CRP Antibody-Coated Plate, 96 wells per plate</td>
<td>1 plate</td>
</tr>
</tbody>
</table>
SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate covers, adhesive strips.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.
9. 37°C incubator.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. Microtiter plates should be allowed to come to room temperature before opening the foil bags. Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
2. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
3. It is recommended that all standards, controls and samples be run in duplicate.
4. Samples that are greater than the highest standard point should be diluted and retested.
5. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
6. Cover or cap all reagents when not in use.
7. **Do not mix or interchange different reagent lots from various kit lots.**
8. Do not use reagents after the kit expiration date.
9. Read absorbances within 30 minutes of assay completion.
10. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
11. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. *Never* insert absorbent paper directly into the wells.

**SAFETY**

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with the *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.
SAMPLE PREPARATION

Specimens should be collected by venipuncture into serum collection tubes. Blood samples may be kept for up to 24 hours before separation of serum. However, it is best to remove serum from the clot as soon as possible after collection. In general, serum may be stored at 2 to 8°C for up to 24 hours or stored frozen at –20°C for longer periods without loss of CRP. It is important that all refrigerated samples are brought to room temperature and mixed to assure accurate determination of the CRP concentration.

All samples should be diluted 1:500 in 1x Assay Diluent prior to assay by addition of 10 μL of sample to 5 mL 1x Assay Diluent.

Do not use grossly hemolysed or lipemic samples.

REAGENT PREPARATION AND STORAGE

1. Assay Diluent

Dilute 1 volume of Assay Diluent Concentrate (20x) with 19 volumes of distilled water. Store both the Assay Diluent Concentrate and 1x Assay Diluent at 2 to 8°C. Diluted Assay Diluent is stable for up to 2 weeks when stored at 2 to 8°C.

2. Wash Buffer

Allow the Wash Buffer Concentrate (20x) to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of Wash Buffer Concentrate (20x) with 19 volumes of distilled water. Store both the Wash Buffer Concentrate and working Wash Buffer at 2 to 8°C. Diluted Wash Buffer is stable for up to 2 weeks when stored at 2 to 8°C.
3. C-Reactive Protein Standard

Add 250 µL of 1x Assay Diluent to each of 6 tubes. Label tubes 120, 60, 30, 15, 7.5 and 0 ng/mL CRP. Add 250 µL C-Reactive Protein Standard to tube labeled 120 ng/mL CRP. Mix well. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

<table>
<thead>
<tr>
<th>Standard:</th>
<th>Add:</th>
<th>Into:</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 ng/mL</td>
<td>250 µL of the C-Reactive Protein Standard supplied</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
<tr>
<td>60 ng/mL</td>
<td>250 µL of the 120 ng/mL Std.</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
<tr>
<td>30 ng/mL</td>
<td>250 µL of the 60 ng/mL Std.</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
<tr>
<td>15 ng/mL</td>
<td>250 µL of the 30 ng/mL Std.</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
<tr>
<td>7.5 ng/mL</td>
<td>250 µL of the 15 ng/mL Std.</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
<tr>
<td>0 ng/mL (Blank)</td>
<td>--</td>
<td>250 µL of the 1x Assay Diluent</td>
</tr>
</tbody>
</table>

The range provided represents a CRP concentration of 3.75 – 60 µg/mL when a sample dilution of 1:500 is taken into account.

Discard all remaining diluted standards after completing assay.
ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the Procedural Notes/Lab Quality Control section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. (Re-bag extra strips and frame, seal bag and store at 2 to 8°C.)
2. Add 100 µL of the diluted sample or standard, in duplicate, to each well.
3. Cover the plate with a plate cover and incubate the plate for 15 minutes at 37°C.
4. After incubation, aspirate or decant solution from wells and discard. Wash the plate 4 times with 1x Wash Buffer. After the last wash, tap the plate dry on absorbent paper. See DIRECTIONS FOR WASHING.
5. Add 100 µL of Anti-Canine CRP Conjugate to each of the wells.
6. Cover the plate with a plate cover and incubate the plate for 15 minutes at 37°C.
7. After incubation, aspirate or decant and wash the plate 4 times with 1x Wash Buffer. See DIRECTIONS FOR WASHING.
8. Add 100 µL of TMB Substrate.
9. Cover the plate with a dust cover and incubate the plate for 15 minutes at room temperature, in the dark.
10. Add 100 µL of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
11. Read the absorbance of each well at 450 nm using 630 nm as a reference wavelength. Read the plate within 30 minutes after adding the *Stop Solution*.

12. Calculate the mean absorbance for each sample, control or standard.

13. Plot the absorbance of the standards against the standard concentration on **linear** or **semi-logarithmic** graph paper. (Optimally, the background absorbance for the blank may be subtracted from each of the data points, including the standards, unknowns and controls prior to plotting.) Draw the best smooth curve through these points to construct the standard curve.

14. Determine the concentrations of the test samples and controls from the standard curve by multiplying the interpolated value by the appropriate dilution factor. Samples that have a signal greater than the top standard, or fall on the non-linear part of the curve, should be further diluted in *Assay Diluent* and reanalyzed.
TYPICAL DATA

An example of a typical standard curve is represented below. This should not be used in the determination of canine CRP.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

Analytical sensitivity is determined by the addition of two standard deviations to the mean of the zero standard assayed 40 times and has been estimated as <1 ng/mL.
PRECISION

1. Intra-Assay Precision

Two samples containing medium and low levels of canine CRP were assayed in replicates of 16 in a single assay. To establish intra-assay reproducibility, the mean and the coefficient of variation (%CV) were calculated.

<table>
<thead>
<tr>
<th>Control</th>
<th>Low</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Mean (µg/mL)</td>
<td>15.4</td>
<td>35.8</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.1</td>
<td>2.3</td>
</tr>
<tr>
<td>%CV</td>
<td>6.9</td>
<td>6.5</td>
</tr>
</tbody>
</table>

2. Inter-Assay Precision

Two samples containing medium and low levels of Canine CRP were run in 32 independent assays. To determine inter-assay reproducibility, the mean and the coefficient of variation (%CV) were calculated.

<table>
<thead>
<tr>
<th>Control</th>
<th>Low</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Mean (µg/mL)</td>
<td>14.9</td>
<td>34.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>%CV</td>
<td>8.2</td>
<td>7.8</td>
</tr>
</tbody>
</table>
LINEARITY OF DILUTION

Purified canine CRP was serially diluted and analyzed with the ELISA. Final concentration of each sample was then multiplied by the appropriate dilution factor. In addition, two canine CRP samples were serially diluted and analyzed. The level of CRP in each dilution was multiplied by the dilution factor. All samples were linearly diluted. Data are presented in table below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Final Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified CRP</td>
<td>1:500</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>1:2000</td>
<td>78.4</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>68.5</td>
</tr>
<tr>
<td>Canine Serum 1</td>
<td>1:500</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>1:2000</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>13.9</td>
</tr>
<tr>
<td>Canine Serum 2</td>
<td>1:2000</td>
<td>53.2</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>52.65</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>53.23</td>
</tr>
<tr>
<td></td>
<td>1:16000</td>
<td>56.63</td>
</tr>
<tr>
<td></td>
<td>1:32000</td>
<td>56.43</td>
</tr>
</tbody>
</table>
SPECIFICITY

Samples with high CRP levels were run on SDS and electro-blotted onto nitrocellulose membrane. Antibodies used in the test reacted with a single band which correlated with the presence of CRP.
CLINICAL VALIDATION

While “accuracy” is generally interpreted as how well a test detects the true level of an analyte, clinical reliability of the test is the most realistic way of assessing accuracy, i.e., does the test give a true picture of the clinical status at time of sampling. The data provided in the figure below are taken from a study that investigated the acute phase response in dogs before and after surgery. The levels of CRP, as well as other canine acute phase proteins, haptoglobin and Serum Amyloid A, were all monitored. In addition, other biological markers, including white blood cells, were also investigated to establish that inflammation was occurring. The resulting CRP profile gives a true picture of the health status/inflammation of the dog before and after surgery.
REFERENCES


Canine CRP Assay Summary

Sample type: Standards
Add 100 µL Standards

Sample type: Control/Serum
Add 100 µL Controls/Sample (serum/1:500 pre-diluted)

↓
Incubate for 15 minutes at 37°C
↓
aspirate and wash 4x

Incubate 100 µL of Anti-canine CRP Conjugate Working Solution for 15 minutes at 37°C
↓
aspirate and wash 4x

Incubate 100 µL of TMB Substrate for 15 minutes at RT
↓
Add 100 µL Stop Solution and read at 450 nm

Total time: 45 minutes

- CRP
- Anti-canine CRP conjugate
- TMB Substrate
- Anti-canine CRP