CAT α-1-ACID GLYCOPROTEIN (AGP) SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: AGP-SP-8

INTRODUCTION

AGP is a highly glycosylated ~40 kDa acute phase protein that is elevated in cat serum as a result of injury, infection or disease. Kajikawa, et al. (ref 1) reported a 19-fold increase of AGP in serum from hospitalized cats and a 5.7-fold increase after injection of normal cats with lipopolysaccharide. Of the positive acute phase proteins investigated (AGP, SAA and CRP), AGP was the most responsive. More recently, Paltrinieri et.al., reported AGP to be a powerful discriminating biomarker for diagnosis of feline infectious peritonitis (ref 2). In studies at Life Diagnostics, we found AGP levels of 346±42 μg/ml in plasma from healthy cats.

PRINCIPLE OF THE ASSAY

The cat AGP SPARCL™[1] (ref 3) assay uses two cat AGP-specific antibodies. One is conjugated to horseradish peroxidase (HRP), the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to AGP they are brought into close proximity. With the addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence. Acridan conjugated antibodies distant from HRP produce no signal. This principle allows the development of a homogeneous assay that allows rapid measurement of AGP concentrations.

The HRP and acridan conjugated antibodies provided with the kit are mixed with standards and diluted samples in wells of the 96-well SPARCL™ plate provided with the kit[2]. After incubation for 30 minutes on a shaker at 25°C and 150 rpm, the plate is placed into a luminometer. Trigger solution containing hydrogen peroxide is injected into each well and luminescence is immediately measured. The concentration of AGP is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-cat AGP HRP conjugate. **Store ≤ -70°C**
- Anti-cat AGP acridan conjugate. **Store ≤ -70°C**
- Cat AGP stock (3 vials). **Store ≤ -70°C**
- Diluent (CSD50-1), 2 x 50 ml
- Trigger solution, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

- Precision pipettes and tips
- Polypropylene tubes
- Vortex mixer
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- Luminometer capable of simultaneous injection and measurement
- PC graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and AGP stock at -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 2-8°C. The SPARCL™ plate should be kept in a sealed bag with desiccant and antioxidant. The kit will remain stable for at least six months from the date of purchase, provided that the components are stored as described above.

GENERAL INSTRUCTIONS

The dilution buffer and 8-well strips used in the assay should be allowed to reach room temperature (25°C) before use.

STANDARD PREPARATION

The cat AGP stock is comprised of lyophilized cat AGP in a carrier protein matrix. The AGP content was determined by reference to purified cat AGP prepared at Life Diagnostics, Inc.

1. Reconstitute the lyophilized cat AGP stock with diluent as described on the vial label. Mix gently until dissolved.
2. Label 8 polypropylene tubes[3] as 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 ng/ml.
3. Into the tube labeled 125 ng/ml, pipette the volume of diluent detailed on the stock vial label. Then add the indicated volume of stock and mix gently. This provides the 125 ng/ml standard.
4. Dispense 150 μl of diluent into the tubes labeled 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 ng/ml.
5. Pipette 150 μl of the 125 ng/ml AGP standard into the tube labeled 62.5 ng/ml and mix. This provides the 62.5 ng/ml AGP standard.
6. Similarly prepare the remaining standards by two-fold serial dilution.

Please Note: Use the standards within one hour of preparation.

SAMPLE PREPARATION

Serum or heparinized plasma should be prepared as quickly as possible after blood collection. If samples cannot be assayed immediately they should be frozen at or below –20°C. Avoid repeated freeze-thaws.

The cat AGP SPARCL™ assay uses a homogeneous format and is therefore susceptible to a prozone or "hook effect" at high AGP concentrations. We found that if samples were tested a dilutions of 4,000-fold or greater, prozone and matrix effects were avoided. We recommend that samples initially be tested at a dilution of 16,000-fold:

1. For each sample to be tested, dispense 198 μl and 318 μl of diluent into separate tubes.
2. Pipette and mix 2.0 μl of sample into the tube containing 198 μl of diluent. This provides a 100-fold dilution.
3. Mix 2.0 μl of the 100-fold diluted sample with the 318 μl of diluent in the second tube. This provides a 16,000-fold dilution.

[1] The SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) technology was developed by Lumigen Corp.

[2] The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

[3] Dilutions of standards can be performed in wells A1-A8 of the clear untreated 96-well plate provided with the kit. This allows rapid transfer of standards to the white SPARCL™ plate using a multipipettor. Diluted samples can also be first aliquoted into appropriate wells of the clear polystyrene plate and subsequently transferred to the SPARCL™ plate with a multipipettor. If using this method, ensure that an excess

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. If necessary, after thawing, briefly centrifuge to ensure that the contents are at the bottom of the tubes. Prepare the mix shortly before use using the diluent provided with the kit.

LUMINOMETER SETUP

1. The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
2. Prime the luminometer injection port with 1 ml of trigger solution.
3. Place the injection needle into the injection port as needed for BMG luminometers.
4. Program the luminometer to inject 37.5 µl of trigger solution per well and to measure from time zero for 1 second (50 x 0.02 second intervals).
5. Define the format of the assay using the luminometer software.
6. Because the white SPARCL™ plate is provided as a 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the luminometer is programmed to inject trigger solution only into the wells being used.
7. We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
8. There are a number of manufacturers of luminometers that are equipped to run a SPARCL™ assay. Please contact Life Diagnostics or Lumigen (www.lumigen.com) to discuss your luminometer.

PROCEDURE

1. Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
2. Secure the desired number of SPARCL™ 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant and antioxidant. Store unused strips at 2-8°C.
3. Aliquot 25.0 µl of conjugate mix into each well.
4. Dispense 50.0 µl of standards[4] and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
5. Incubate on an orbital micro-plate shaker at 150 rpm 25°C for 30 minutes.
6. After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 µl).
7. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

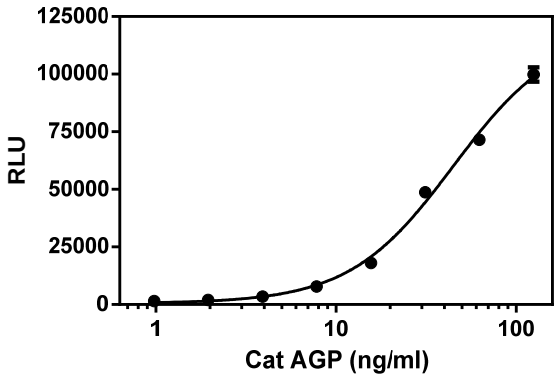
CALCULATION OF RESULTS

1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells (we use RLU values acquired from 100-980 ms).
2. Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus Log_{10} of the AGP concentration.
3. Fit data using a sigmoidal four parameter logistic equation.
4. Derive the corresponding concentration of AGP in the samples from the standard curve (convert the antilog to concentration in ng/ml).
5. Multiply the derived concentration by the dilution factor to determine the actual concentration of AGP in the serum or plasma sample.
6. If the RLU values of diluted samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with RLU plotted on the Y-axis versus AGP concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns.

AGP(ng/ml)	RLU
125	99829
62.5	71538
31.25	48692
15.63	18051
7.81	7862
3.91	3468
1.95	2009
0.98	1559



REFERENCES

1. Kajikawa, et al. Changes in concentrations of serum amyloid A protein, α-1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Vet Immunol Immunopathol. 68(1):91-98 (1999)
2. Patrinieri S, etal. Critical assessment of the diagnostic value of feline a1-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach. J Vet Diagn Invest. 19:266-272 (2007)
3. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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volume is aliquoted into the clear plate in order to ensure complete transfer of 50 µl aliquots to the SPARCL™ plate.

[4] A standard curve must be generated for each experiment.