INTRODUCTION

Alpha-1-acid glycoprotein (AGP) is a highly glycosylated acute phase protein. Serum concentrations increase during diseases that cause inflammation. It has potential as a biomarker of feline infectious peritonitis (refs 1-2).

PRINCIPLE OF THE ASSAY

The assay uses two cat AGP monoclonal antibody conjugates; one to horseradish peroxidase (HRP), the other to acridan; a chemiluminescent substrate. When HRP and acridan conjugates bind to AGP they are brought into proximity. Upon addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of luminescence that is proportional to AGP concentration.

In practice, a blank, a standard, and diluted samples (10 µl) are pipetted into test tubes and mixed with 0.5 ml of combined acridan and HRP conjugates. After 45 minutes, the tubes are sequentially inserted into the VetBio-1 luminometer. Luminescence is measured after injection of background reducer, followed by a hydrogen peroxide-containing trigger-solution. The concentration of AGP is determined from the ratio of blank-subtracted sample luminescence to the that of the standard. The schematic below illustrates the sequence of events in the AGP VetBio-1 assay.

Equilibrate reagents to room temperature

⇩

Prime the luminometer

⇩

Program the luminometer and save the program

⇩

Dilute samples

⇩

Prepare standard

⇩

Add blank, standard and diluted samples to tubes (10 µl/tube)

⇩

Reconstitute the acridan/HRP conjugate mix (5.5 ml/vial)

⇩

Add conjugate mix to the tubes (0.5 ml/tube)

⇩

Incubate tubes at room temp for 45 min

⇩

Run the Quick Prime program

⇩

Load the program. Test standards and samples

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Acridan & HRP Conjugates, 5 vials. **Store at -20°C**
- AGP Standard, 1 vial. **Store at -20°C**
- Diluent: CSD50-1, 50 ml
- Background Reducer: BR9-1, 9 ml
- Trigger Solution: TS12-1, 12 ml

Materials required but not provided:

- VetBio-1 luminometer
- Precision pipettors and tips
- 12 x 75 mm borosilicate tubes[1]
- 12 x 75 mm test tube racks
- De-ionized water
- 15-ml centrifuge tubes & rack
- 10-ml pipets
- Microcentrifuge tubes

STORAGE

Store the conjugate and AGP standard vials at -20°C. The remainder of the kit should be stored at 4°C.

[1] Only cylindrical 12 x 75 mm borosilicate glass tubes can be used. Do not use polystyrene or polycarbonate tubes.

GENERAL INSTRUCTIONS

1. Please take the time to completely read all instructions before starting your assay**.** Contact us if you need clarification.
2. All reagents used in the assay should be allowed to reach room temperature before use.
3. It is important that the assay is performed in an area free of UV-light (sunlight). Therefore, please close window blinds. UV-light can cause borosilicate glass tubes to phosphoresce, leading to interference in the assay.

LUMINOMETER SETUP

1. Turn the VetBio-1 luminometer on.
2. Place the tubing from injector 1 into the tube containing Background Reducer. Tube holders are positioned adjacent to the injectors.
3. Place the tube from injector 2 into the tube containing Trigger Solution.
4. From the Protocol manager on the keypad select “Prime & Wash,” "Prime," then “Start.”
5. Open the luminometer drawer and insert an empty 12 x 75 mm tube.
6. Close the drawer and press “Start” again.
7. Wait for priming to complete. Open the drawer and discard the tube.
8. From the Protocol manager select “Measure” followed by “Cat AGP.”
9. Select “Edit.”
10. The experiment setup screen will be displayed. Enter the sample dilution, replicates for the blank/standard, replicates for the samples, then press “Start.”
11. Enter the number of samples to be evaluated.
12. Enter the sample IDs and dilution factor(s), if different than the default dilution. You can also enter experimental comments.
13. After entering information, press save. The experiment will be saved as a “prepared experiment.”

SAMPLE DILUTION

We found AGP levels ranging from ~200 μg/ml in healthy cats to >1500 μg/ml in sick cats. We suggest that samples be evaluated at a dilution of 1000-fold to obtain values within range of the standard. A 1000-fold dilution can be obtained as follows.

1. Dispense 96 μl and 195 μl of diluent into separate microcentrifuge tubes.
2. Mix 4 μl of serum with 96 μl of diluent in the first tube. This gives a 25-fold dilution.
3. Mix 5 μl of the 25-fold diluted sample with 195 μl of diluent in the second tube. This gives a 1000-fold dilution.

STANDARD PREPARATION

1. The AGP standard is provided in lyophilized form.
2. Add the volume of diluent indicated on the vial label and mix. Use a vortex mixer if available. This provides a 1000 ng/ml standard.
3. Pipet 10.0 ul into tube 2 as described in procedure section.

If stored in a sealed vial, the reconstituted standard is stable for at least two days at room temperature (> 1 week at 4°C). It may also be aliquoted and stored frozen at or below -20°C.

CONJUGATE PREPARATION

1. The acridan and HRP conjugate mixture should be prepared just before use.
2. Tap the vial to ensure that the contents are at the bottom of the vial before gently removing the stopper (it is under vacuum).
3. Add 5.5 ml of diluent to the vial. Insert the stopper and mix gently by inversion at least ten times.
4. Each vial of reconstituted conjugate mix provides enough reagent to measure the blank, standard, and up to eight samples.[2]

Unused conjugate should be promptly frozen at -20°C in a sealed vial if future use is intended. Thaw once only.

PROCEDURE

STEP 1

1. Determine the number of 12 x 75 mm borosilicate glass tubes required (one each for the blank, standard, and the samples). Ensure that all the tubes you intend to use fit easily in the VetBio-1 sample holder before beginning the test.
2. Pipet 10 μl of diluent into tube 1. This serves as the zero standard or blank.
3. Pipet 10 μl of the 1000 ng/ml AGP standard into tube 2.
4. Pipet 10 μl of the diluted samples into tubes 3, 4, 5…
5. Add 0.50 ml of freshly prepared conjugate mix to each tube and mix gently. A vortex mixer may be used if available. Avoid creating bubbles.
6. Incubate the mixtures at room temperature for 45 minutes.

STEP 2

1. Two to three minutes before the end of the incubation step, insert an empty tube into the sample holder. Select “QuickPrime” from the Protocol manager, close the drawer and press start. Discard the tube after completion. This step ensures that the injectors are fully loaded.
2. Recall your saved experiment and press start.
3. After the end of the incubation, insert tube 1 (Blank) into the sample holder of the luminometer and close the drawer. When the luminescence value is recorded on the screen open the drawer and discard the tube.
4. Similarly measure luminescence of the standard (tube 2), followed by the samples (tubes 3, 4, 5…).
5. AGP concentrations of the samples are automatically calculated.

[2] If using multiple vials of conjugates to measure more than eight samples, combine the reconstituted contents of all vials and mix briefly before dispensing 0.5 ml aliquots into the reaction tubes.

6. After measurement of the last sample, select “End.”
7. Results will be saved and can be recalled. They may be exported as Excel or pdf files via a USB stick.

RESULTS

If RLU/s values for diluted samples are between the blank and standard, assume that the calculated values are correct. If RLU/s values significantly exceed that of the standard (i.e., twice the standard RLU/s value), AGP concentrations may be underestimated. If an accurate measurement is required, such samples should be retested at a higher dilution.

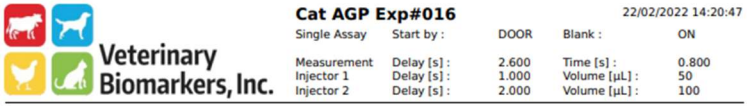
LUMINOMETER MAINTENANCE

The luminometer injectors must be cleaned with distilled or deionized water at the end of each day that the instrument is used to avoid clogging.

1. From the Protocol manager screen select “Prime & Wash.”
2. Select “Backprime” to return unused reagents to the respective tubes.
3. Cap the background reducer and trigger solution tubes and store them at 4°C.
4. Wipe the tubing from injectors 1 and 2 and place them in separate 15 ml centrifuge tubes containing distilled or deionized water.
5. Select “Wash” from the “Prime & Wash” screen. Press “Start,” insert an empty tube into the luminometer, close the drawer and press “Start.”
6. Discard the tube.
7. Insert an empty tube and repeat steps 5 and 6.
8. Leave the injector tubing immersed in water.
9. Switch the luminometer off. It should not be left powered-up overnight.

ASSAY PERFORMANCE

Typical data: The table below show results from an assay in which eight serum samples were evaluated.



Sample	Dilution	Rep	RLU/s	Conc [µg/mL]
Blank			1,396	0
Standard			113,648	1
9	1000		28,956	245.52
10	1000		18,676	153.94
11	1000		28,615	242.48
12	1000		30,569	259.89
13	1000		2,728	11.86
14	1000		167,909	1,483.4
15	1000		36,501	312.74
16	1000		32,065	273.22

Reproducibility. Four serum samples with different AGP levels were evaluated in triplicate in three separate assays. Levels of AGP are reported as mean±SD.

Intra-assay variability (assay 1)

Sample	Dilution	AGP (µg/ml)
1	500	309.5 ± 3.4
2	500	365.1 ± 7.6
3	1000	1006.3 ± 25.3
4	1000	1282.2 ± 25.3

Inter-assay variability (assays 1-3)

Sample	Dilution	AGP (µg/ml)
1	500	326.4 ± 11.8
2	500	371.7 ± 17.1
3	1000	1063.1 ± 37.4
4	1000	1338.6 ± 24.8

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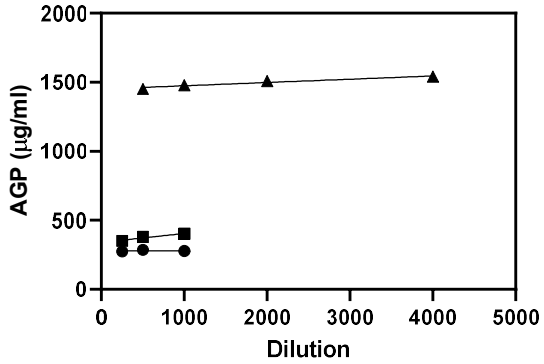
Parallelism. Three serum samples with AGP levels of 278, 378 and 1495 μg/ml, were evaluated at dilutions of 250- to 4000-fold. Parallelism was obtained.



REFERENCES

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2. Saverio P. et al. Critical assessment of the diagnostic value of feline α1-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach. J Vet Diagn Invest 19:266–272 (2007)

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