"PHASE"™ Serum Amyloid A Assay (SAA) – Multispecies Cat. No. TP-802

Intended use

This assay is designed to detect Serum Amyloid A (SAA) in serum or plasma from a range of species. A modified version of this product can also be used to detect Amyloid A in milk (Cat number: TP 807).

Background

The Serum Amyloid A family of acute phase proteins are named because of their immunological and biochemical similarity to Amyloid A, the fibril protein in reactive systemic amyloidosis. The liver produces several different isoforms of SAA following stimulation by immune system modulators including interleukin-1, interleukin-6 and tumor necrosis factor. In its native form, SAA generally consists of a 104-amino-acid polypeptide (12kd) in association with the HDL 3 subclass of plasma lipoproteins.
Circulating SAA concentrations may increase up to 1000-fold following inflammation, infection, tissue injury and cell necrosis and decline rapidly following recovery.

Assay Principle

The Tridelta Phase™ range SAA kit is a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). A monoclonal antibody specific for SAA has been coated onto the wells of the microtitre strips provided. Samples, including calibrators of known SAA content, are incubated in micro-wells at 37°C together with a HRP labelled anti-SAA antibody. Any SAA present will be captured between the coated microplate and the labelled antibody. The plate is washed after sample and antibody-HRP incubation to remove any unbound material. Following the addition of TMB, a blue product is generated in direct proportion to the amount of SAA present in the original sample or calibrator. The reaction is stopped with the addition of stop reagent.

Reagents Provided

1. SAA antibody coated wells	1 x 96 well plate
2. Wash buffer concentrate	1 x 50ml (20x concentrate)
3. Sample/calibrator diluent	1 x 30ml (10x concentrate)
4. SAA Calibrator	1 x freeze dried vial
5. Anti-SAA conjugate	1 x 6ml (Ready to use)
6. TMB substrate	1 x 11ml (Ready to use)
7. Stop reagent	1 x 11ml (Ready to use)

Additional Materials Required

1. Serum/Plasma collection equipment.
2. Microtiter plate reader capable of measurement at 450nm with reference at 630nm if available.
3. Accurate micropipettes and disposable tips to deliver 0-10µl, 20-200µl and 200-1000µl.
4. A repeat or multichannel pipette (50-200µl) for large assays.
5. Deionized or distilled H_2O.
6. Plate washer (Optional).
7. Graph paper: linear (Cartesian).
8. Glass or plastic test tubes.
9. Absorbent paper towels.
10. 96 well dust plate cover.
11. 37°C incubator.
12. Vortex.
13. Centrifuge (Optional)

Precautions

Safety

- For *in vitro* research purposes only.
- Some reagents contain thimerosal and may be toxic if ingested.
- Dispose of all clinical specimens, infected or potentially infectious material in accordance with good laboratory practice. All such materials should be handled and disposed of as though potentially infectious.
- Never pipette by mouth and never eat or drink at the laboratory workbench.
- Wear disposable latex gloves and eye protection where appropriate.
- The stop solution and TMB contain reagents that may irritate the skin or mucous membranes. Any reagent which comes into contact with the skin should be washed off with water immediately.
- Wash hands thoroughly when finished.

Procedural

- Do not use kit or individual reagents past their expiry date.
- Do not mix or substitute reagents from different kit lot numbers.
- Deviation from protocol provided may cause erroneous results.
- Samples should be stored refrigerated or frozen if they are not to be analysed shortly after collection. Avoid repeated freeze thaw cycles.
- When possible avoid the use of badly haemolysed or lipemic sera. If large amounts of particulate matter are present, this should be removed by centrifugation prior to assay.
- It is recommended that all calibrators and samples are run in duplicate.
- Allow all reagents to come to room temperature (20 – 25^{0}C) and mix well before use.
- Avoid leaving reagents in direct sunlight and/or above 4^{0}C for extended periods.
- Cover or cap all reagents when not in use.
- High quality distilled or deionised water is required for the Wash Solution and Diluent Buffer. The use of contaminated water may lead to background interference in the assay.
- Always use clean, preferably disposable, labware for all reagent preparation.
- Care must be taken not to contaminate components and always use fresh tips for each sample and component.
- Reagent delivery should be aimed at the midpoint of the side of microtitration wells, taking care not to scratch the side with the pipette tip.
- Do not allow microwells to dry out at any stage during the procedure. Never insert absorbent paper directly into the wells.
- Ensure that the bottom outer surface of the well is clean and dry before reading.
- Before commencing the assay an identification and distribution plan should be established. It also recommended labelling each strip to enable identification.
- Read absorbance's shortly after completion of the assay.
- SAA values should only be determined from the 'linear portion' of the curve.

Serum or Plasma

Specimen Preparation

Serum /Plasma

Specimens should be collected by venipuncture into serum or plasma collection tubes. Blood samples may be kept for up to 24 hours before separation of serum or plasma. However, it is best to remove serum from the clot or cells and debris from plasma or other fluids as soon as possible after collection. In general, serum or plasma may be stored at 2-8^{o}C for up to 24 hours or stored frozen at –20^{o}C for longer periods without deterioration of SAA. Repeated freeze thaw cycles should be avoided. It is important that all refrigerated samples are brought to room temperature before use. If large amounts of particulate matter are present, this should be removed by centrifugation prior to assay.

Note: Because SAA levels can increase as much as 1000 fold during inflammation, it is recommended that the optimal dilution should be determined empirically.

As a good starting point, all serum or plasma samples (excluding equine) should be diluted 1:500 in 1x diluent buffer (see below for preparation of this buffer) prior to assay by the addition of 10ul of sample to 5.0ml of 1x diluent buffer.

For all equine serum or plasma samples, a good starting dilution should be 1:2000 in 1x diluent buffer prior to assay. Dilute the equine sample 1:10 initially by adding 10ul of sample to 90ul of 1x diluent buffer ('intermediate sample' dilution). To achieve a final 1:2000 dilution, add 10ul of this 'intermediate sample' to 2ml of 1x diluent buffer.

Reagent Preparation

1x Sample/sample diluent (1x diluent buffer, BLUE)

Dilute 1 volume of sample/calibrator diluent concentrate (10x) with 9 volumes of distilled water. Prepared reagent (1x diluent buffer) is stable for one day at room temperature.

1x Wash buffer (RED)

Dilute 1 volume of wash buffer concentrate (20x) with 19 volumes of distilled water. Store both the wash buffer concentrate and working wash buffer (1x) in the refrigerator. Diluted wash solution is stable for up to 2 weeks when stored at $4^{o}C$.

NOTE: Ensure that any crystals that may have developed in the diluent have been completely dissolved prior to dilution for use. This may be accomplished by incubation the bottle at $37^{o}C$ for several minutes.

Dilution of SAA calibrators

1. To prepare the top calibrator, reconstitute the SAA calibrator provided in the kit by adding 1ml of 1x diluent buffer to the vial. Vortex vigorously to dissolve completely.
2. Label 5 tubes C1-C6. Add 300ul of the top calibrator to the first tube labelled C1. Immediately aliquot (320ul per aliquot) and freeze remaining unused top calibrator material at $-20^{0}C$.
3. Add 150ul of 1x diluent buffer to the remaining 4 tubes labelled C2-C6 respectively, where tube C5 represents the lowest calibrator with SAA and C6 is the zero calibrator or assay blank. (Diluent buffer only).
4. Add 150ul of the top calibrator (C1) to tube C2. Mix well and serially dilute down to complete the range as directed in Table 1.
5. Discard all diluted calibrators immediately after use and prepare a new range as required from the frozen stock calibrator – step 2 above.

Table 1: Preparation of Working Calibration Curve

Tube Number	Volume of kit calibrator (ul)	Volume of 1x diluent buffer(ul)	Serial Dilution
C1	300	--	---
C2	-	150	150 of C1
C3	-	150	150 of C2
C4	-	150	150 of C3

C5	-	150	150 of C4
C6	-	150	-

Table 2: Concentration of calibrators for Serum or Plasma

Standards	Bovine (ng/ml)	Porcine (ng/ml)	Canine (ng/ml)	Feline (ng/ml)	Equine (ng/ml)
C1	300	1000	160	100	20
C2	150	500	80	50	10
C3	75	250	40	25	5
C4	37.5	125	20	12.5	2.5
C5	18.8	62.5	10	6.25	1.25
C6	0	0	0	0	0

Procedure

Allow test reagents and samples to reach room temperature before use.

1. Prepare appropriate volumes of assay reagents as described above under 'reagent preparation'.
2. Ensure the serum or plasma samples are homogenous before use. Dilute serum and plasma samples 1:500 (equine samples 1:2000) in 1x diluent buffer. If large amounts of particulate matter are present, this should be removed by centrifugation prior to assay.
3. Prepare the calibration curve as outlined in table 1 above.
4. Determine the number of 8-well strips needed for the assay. Re-bag extra strips, seal bag and store in a refrigerator.
5. Add **50µl** of Anti-SAA/HRP (yellow) conjugate to each well.
6. Add **50µl**, in duplicate, of diluted calibrator, control or sample to each well. Tap sides of the plate gently to mix.
7. Cover the plate with a dust cover. Incubate the plate for **1 hour at 37°C**.
8. After incubation aspirate or decant and wash the plate four times with diluted wash buffer. After the last wash, tap the plate dry on absorbent paper.
9. Add **100µl** of TMB substrate
10. Cover the plate and incubate at **room temperature for 15 minutes**.
11. Add **100µl** of stop solution and tap gently to mix.
12. Read the absorbance of each well at 450nm using 630nm as a reference, if available.

Summary of Assay Procedure

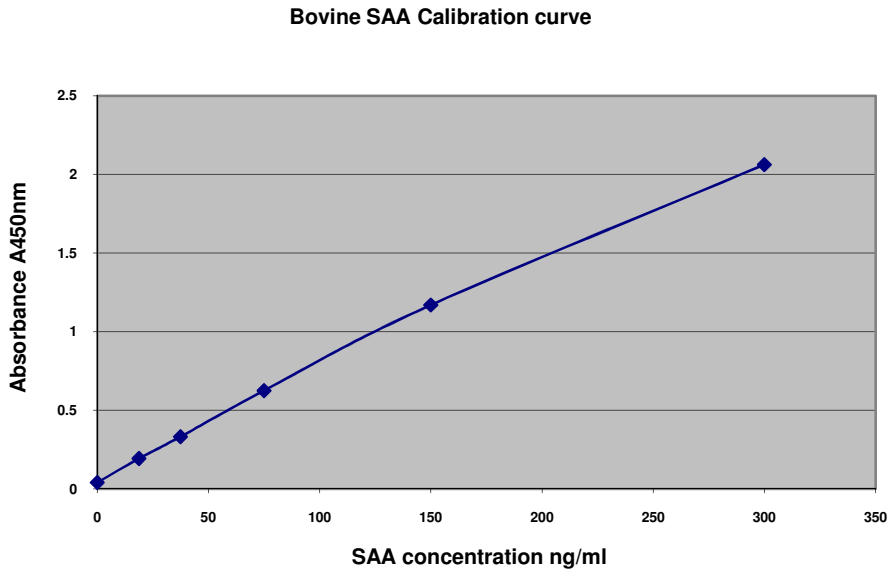
Summary SAA ELISA
Coated microplate: 96 well
50 µl of Anti-SAA/HRP conjugate (Ready to use) + **50 µl** calibrator, control or sample (1:2000 equine and 1:500 all other species)
Incubate 1 hour at 37^0C
Wash plate 4 times
100 µl TMB substrate
Incubate 15 min at room temperature in the dark
100 µl stop solution (Do NOT wash)
Read absorbance at 450 nm

Interpretation of Test Results

1. Calculate the mean absorbance for each sample, control or standard.
2. Plot the absorbance of the standards against the calibrator concentration on **semi-logarithmic or standard** graph paper. (If necessary, the background absorbance for the 0ng/ml blank calibrator may be subtracted from each of the data points, including the calibrators, test samples and controls prior to plotting). Draw the best smooth curve through these points to construct the calibration curve.
3. Determine the concentrations of the test samples and controls from the calibration curve by multiplying the interpolated value by the appropriate dilution factor (eg, serum or plasma diluted 1:500 should be multiplied by 500). Samples that have a signal greater than the top calibrator or fall on the non-linear part of the curve should be further diluted in 1x diluent buffer and re-analysed.

Typical Data

An example of a typical calibration curve is represented below. This should not be used in the determination of SAA each user should obtain his or her data and standard curve in each experiment. A calibration curve must be run with each assay.



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Performance Characteristics

A) Measuring Range

For serum or plasma the measuring range will depend on the species under investigation.

Bovine: 9.4 - 150 ug/ml
Porcine: 31.25 - 500 ug/ml
Canine: 5.0 - 80 ug/ml
Feline: 3.125 - 50 ug/ml
Equine: 2.5 - 40 ug/ml

Note: These measuring ranges are calculated using the minimum recommended dilution (1:500) for each species (1:2000 for equine use).

B) Intra assay reproducibility data

Two bovine serum samples containing different levels of SAA were diluted 1:2000 and assayed in replicates (16 times) to determine intra (within) assay precision/reproducibility.

Intra (within) assay reproducibility

	Level 1 (µg/ml)	**Level 2 (µg/ml)**
n	16	16
Mean (ug/ml)	143.19	245.56
Standard Deviation	7.19	11.07
%CV	5.0	4.5

Inter batch reproducibility data

Two bovine serum samples containing different levels of SAA were diluted 1:2000 and assayed in replicates (16 times) in three consecutive production batches to determine **inter – batch** precision/reproducibility.

Inter batch assay reproducibility

	Level 1 (µg/ml)	**Level 2 (µg/ml)**
n	48	48
Mean (ug/ml)	127.73	242.2
Standard Dev	14.55	15.07
%CV	11.4	6.22

C) Analytical Sensitivity

For serum or plasma the sensitivity will depend on the species under investigation. Sensitivities are 0.8ug/ml, 0.3ug/ml, 0.15ug/ml and 0.02ug/ml for porcine, bovine/feline, canine and equine samples respectively. These values were determined by the addition of two standard deviations of the mean OD obtained when the zero calibrator was assayed 32 times.

D) Limitations of the procedures

Serum or plasma samples are recommended for use in this test. However to eliminate potential discrepancies it is recommended that any study which starts with a particular matrix, i.e. serum or plasma, should continue to use the same matrix for the duration of the investigation.

The test can also be used to detect SAA in cell culture medium. Details are available on request from Tridelta Development Ltd.

Other "PHASE" acute phase assays available from Tridelta:

TP-801	Haptoglobin	Colormetric rapid test that can be used in a manual method or on a wide range of auto analysers. (Settings for some popular analysers available on request)
TP-801-Cal	Haptoglobin	Haptoglobin *calibrator* (2mg/ml) for Haptoglobin kit (TP-801).
TP-801-Con	Haptoglobin	Haptoglobin *controls* for Haptoglobin kit (TP-801).
TP-803	CRP-Canine	EIA C-reactive Protein assay specific for canine.
TP-803-Con	CRP-Canine	Canine CRP *controls* for Canine CRP EIA kits (TP-803).
TA-901	CRP-Porcine	EIA C- reactive Protein assay specific for porcine.
TP-802M	Murine SAA	EIA Serum Amyloid A assay specific for mouse.
TP-802M-CON	Murine SAA	M-SAA *controls* for Murine SAA Kit (TP-802M).
TP-807	Milk MAA	EIA Amyloid A specific for milk.
TP-802-CON	Multi-species SAA	SAA *controls* for Multi-species SAA Kit (TP-802).

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