MOUSE SAA-3 ELISA KIT PROTOCOL
96-Well Plate (Cat. # EZMSAA3-12K)

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MOUSE SAA-3 ELISA KIT
96-Well Plate (Cat. # EZMSAA3-12K)

I. INTENDED USE

This kit is used for the non-radioactive quantification of Mouse SAA-3 in serum, plasma and other biological media. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE[1]

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of Mouse SAA-3 from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-Mouse SAA-3 polyclonal antibody, 2) wash away of unbound materials from samples, 3) binding of a biotinylated anti-Mouse SAA-3 polyclonal antibody to the captured Mouse SAA-3, 4) wash away of unbound materials from samples, 5) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured Mouse SAA-3 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Mouse SAA-3.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well microtiter plate and contains the following reagents:

A. Mouse SAA-3 ELISA Plate

Coated with Rabbit anti-Mouse SAA-3 Antibody
Quantity: 1 strip plate
Preparation: Ready to use
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer

Quantity: 2 Sheets
Preparation: Ready to use

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween 20
Quantity: Two bottles containing 50 mL each
Preparation: Dilute 1:10 with deionized water

III. REAGENTS SUPPLIED (continued)

D. Mouse SAA-3 Standard

Purified Recombinant GST-tagged Mouse SAA-3, lyophilized.
Quantity: 0.25 mL/vial upon hydration
Preparation: Reconstitute with 0.25 mL distilled or deionized water. See insert for concentration,

E. Mouse SAA-3 Quality Controls 1 and 2

One vial each , lyophilized, containing Mouse SAA-3 at two different levels
Quantity: 0.25 mL/vial upon hydration
Preparation: Reconstitute with 0.25 mL distilled or deionized water.

F. Assay Buffer

0.05M PBS, pH 7.4, containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA and 0.05% Triton X-100
Quantity: Two bottles containing 10 mL each
Preparation: Ready to use

G. Mouse SAA-3 Detection Antibody

Pre-titered Biotinylated Mouse SAA-3 Antibody
Quantity: 11 mL/vial
Preparation: Ready to use

H. Enzyme Solution

Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mLl/vial
Preparation**:** Ready to use

I. Substrate

3,3'5,5'-tetramethylbenzidine
Quantity: 12 mL
Preparation: Ready to use

J. Stop Solution

0.3M HCL
Quantity: 12 mL/vial
Preparation: Ready to use **(Caution: Corrosive Material)**

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8^{o}C. For longer storage (> 2 weeks), freeze Wash Buffer, Assay Buffer, and reconstituted Standards and Controls at ≤ –20^{o}C. Minimize repeated freeze and thaw of the SAA-3 Standards and Quality Controls. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers

V. REAGENT PRECAUTIONS

A. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

B. Sodium Azide

Sodium Azide has been added to some reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipette with Tips, 10µL-200µL
2. Multi-channel Pipette, 50µL-300µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Absorbent Paper or Cloth
6. Deionized Water
7. Microtiter Plate Reader capable of reading absorbency at 450 nm
8. Orbital Microtiter Plate Shaker

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum, whole blood is directly drawn into a glass tube that contains no anti-coagulant. Let blood clot at room temperature for 30 minutes.
2. Promptly centrifuge the clotted blood at 2000 to 3000 x.g. for 15 minutes at 4 ± 2ºC.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Avoid multiple (>5) freeze/thaw cycles.
5. To prepare plasma samples, whole blood should be collected into EDTA-plasma tubes and centrifuged immediately after collection. Observe same precautions in the preparation of serum samples.
6. If heparin is to be used as an anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

VIII. REAGENT PREPARATION

A. Mouse SAA-3 Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Mouse SAA-3 Standard with 0.25 mL distilled or deionized water to give a concentration described in the analysis sheet. Invert and mix gently, let sit for 5 minutes then vortex gently.

2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.1 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.1 mL of the reconstituted standard to Tube 1, mix well and transfer 0.1 mL of Tube 1 to Tube 2, mix well and transfer 0.1 mL of Tube 2 to Tube 3, mix well and transfer 0.1 mL of Tube 3 to Tube 4, mix well and transfer 0.1 mL of Tube 4 to Tube 5, mix well and transfer 0.1 mL of Tube 5 to Tube 6 and mix well.

   Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration (µg/mL)
0.25 mL	0	X (refer to analysis sheet For exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (µg/mL)
Tube 1	0.1 mL	0.1 mL of reconstituted standard	X/2
Tube 2	0.1 mL	0.1 mL of Tube 1	X/4
Tube 3	0.1 mL	0.1 mL of Tube 2	X/8
Tube 4	0.1 mL	0.1 mL of Tube 3	X/16
Tube 5	0.1 mL	0.1 mL of Tube 4	X/32
Tube 6	0.1 mL	0.1 mL of Tube 5	X/64

B. Mouse SAA-3 Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Mouse SAA-3 Quality Control 1 and Quality Control 2 with 0.25 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

IX. ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated Wash Buffer 10 fold by adding the entire contents of one bottle of wash buffer concentrate to 450 mL de-ionized or distilled water (dilute both buffer bottles with 900 mL deionized or distilled water).

2. Remove required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Assemble strips in an empty plate holder and add 300 µL of diluted Wash Buffer to each well. Incubate at room temperature for 5 minutes. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 90 µL Assay Buffer into all wells.

4. Add in duplicate 10 µL Assay Buffer to blank wells. (Refer to Section IX for suggested well Orientations.)

5. Add in duplicate 10 µL Mouse SAA-3 Standards in order of ascending concentration to the appropriate wells. Add in duplicate 10 µL QC1 and 10 µL QC2 to the appropriate wells. Add sequentially 10 µL of samples in duplicate to the remaining wells**. For best results all additions should be completed within 30 minutes**.

6. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

7. Remove plate sealer and decant solutions from the plate.Tap as before to remove residual solutions in the wells.

8. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

9. Add 100 µL Detection Antibody to each well. Cover the plate with sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400-500 rpm.

10. Remove sealer and decant solution from the plate. Tap as before to remove residual solutions in the wells.

11. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

IX. ASSAY PROCEDURE continued)

12. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

13. Remove sealer, decant solution from the plate, and tap plate to remove the residual fluid.

14. Wash wells 6 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

15. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for 5 - 20 minutes (A longer development time may be needed if using a plate washer). Blue color should be formed in wells of SAA-3 standards with intensity proportional to increasing concentrations of SAA-3.

    NOTE: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

16. Remove sealer and add 100 µL of Stop Solution (**Caution: Corrosive solution**) and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450nm and 590nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference in absorbance units. The absorbance of the highest SAA-3 standard should be approximately 1.5 – 2.2, or not to exceed the capability of the plate reader used.

Assay Procedure for Mouse SAA-3 ELISA kit (Cat. # EZMSAA3-12K)

	Step 1	Step 2	Step3-4	Step 5	Step 6-8	Step 9	Step 9-11	Step 12	Step 12-14	Step 15	Step 15	Step 16	Step 16
Well #	Dilute 2 bottles of 10X Wash Buffer with 900mL Deionized Water.	Add 300 µl Wash Buffer to each well and incubate at room temperature for 5 minutes. Remove residual buffer by tapping smartly on absorbent towels	Assay Buffer	Standards/ Controls/ Samples	Seal, Agitate, Incubate 2 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer	Detection Ab	Seal, Agitate, Incubate 1 hour at Room Temperature. Remove residual buffer by tapping smartly on absorbent towels. Wash 3X with 300 µL Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 6X with 300 µLWash Buffer	Substrate	Seal, Agitate, Incubate 5-20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			100 µL	---		100 µL		100 µL		100 µL		100 µL	
C1, D1			90 µL	10 µL of Standard #6									
E1, F1				10 µL of Standard #5									
G1, H1				10 µL of Standard #4									
A2, B2				10 µL of Standard #3									
C2, D2				10 µL of Standard #2									
E2, F2				10 µL of Standard #1									
G2, H2				10 µL of Original Standard									
A3, B3				10 µL of QC 1									
C3, D3				10 µL of QC 2									
E3, F3				10 µL of Sample									
G3, H3				10 µL of Sample									
G4, H4 ↓				10 µL of Sample									

X. MICROTITER PLATE ARRANGEMENT

Mouse SAA-3 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3 Standard	QC 1	Etc.								
B	Blank	Tube 3 Standard	QC 1									
C	Tube 6 Standard	Tube 2 Standard	QC 2									
D	Tube 6 Standard	Tube 2 Standard	QC 2									
E	Tube 5 Standard	Tube 1 Standard	Sample 1									
F	Tube 5 Standard	Tube 1 Standard	Sample 1									
G	Tube 4 Standard	Original Standard	Sample 2									
H	Tube 4 Standard	Original Standard	Sample 2									

XI. CALCULATIONS

The dose-response curve of this assay fits best to a 4 or 5-parameter logistic equation. The results of unknown samples using GST-tagged Mouse SAA-3 as standard can be calculated with any computer program having a 4 or 5-parameter logistic function.

Note: When sample volumes assayed differ from 10 µL (in normal assay), an appropriate mathematical adjustment must be made to accommodate for additional dilution factor (e.g., if 5 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 10 µL, compensate the volume deficit with assay buffer.

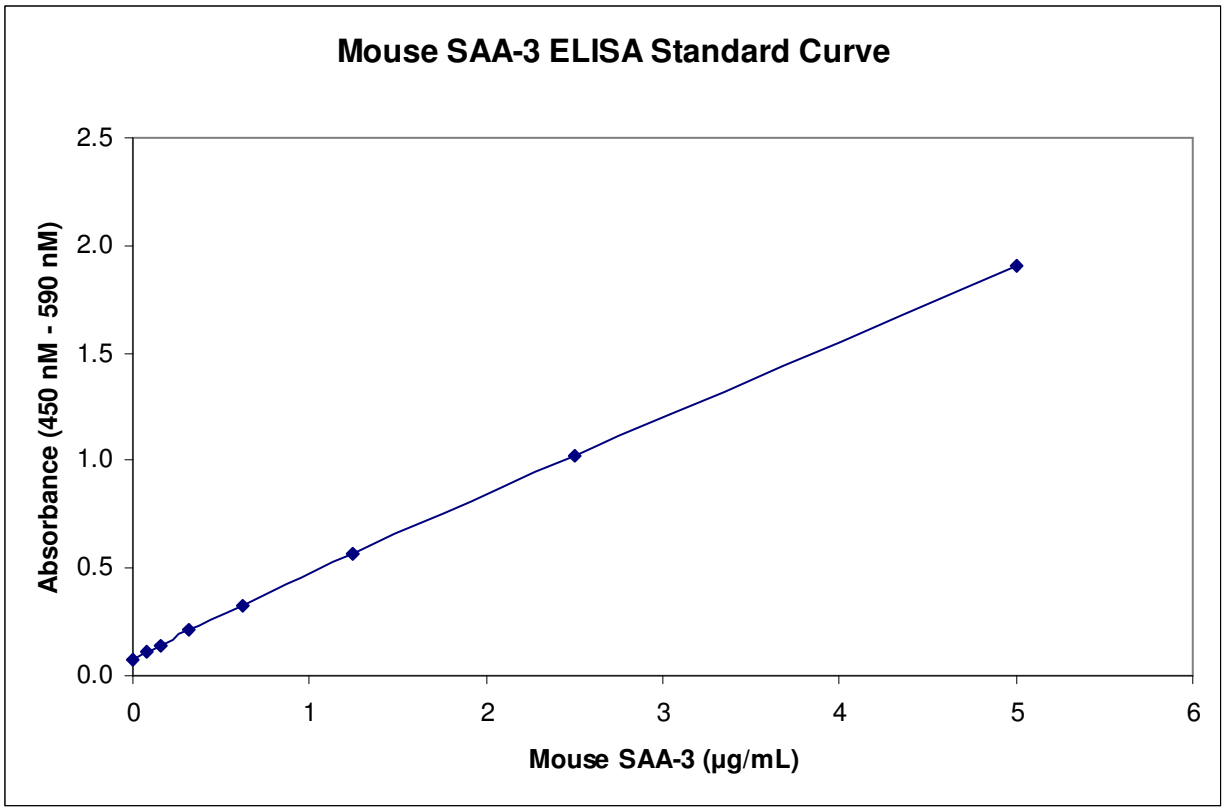
XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity of this assay is 0.078 µg/mL Mouse SAA-3 (10 µL sample size).
4. The appropriate range of this assay is 0.078 to 5 µg/mL Mouse SAA-3 (20 µL sample size). Any result greater than 5 µg/mL in a 10 µL sample assayed should be diluted and repeated using assay buffer as diluent until it falls within range.

XIII. NORMAL RANGE

SAA-3 levels in "normal" CD-1 mouse range from 0.1 – 1.0 µg/mL.

XIV. STANDARD CURVE



Typical Standard Curve – Not to be used to calculate data

XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Mouse SAA-3 standard used in this assay is 0.078 µg/mL (10 µL sample size).

B. Specificity

Mouse SAA-3	100%
Complement C1q Sigma C1740 10 ug/mL	N.D.
Rat Tail Collagen Type I Sigma C7661 10 ug/mL	N.D.
Hu Placenta Collagen Type VI Sigma C7521 10 ug/mL	N.D.
mPTX-3 0.5 ug/mL	N.D.
24P3 2.5 ug/mL	N.D.
mAGP 1.0 ug/mL	N.D.
mAdipsin 2.5 ug/mL	N.D.
GST protein 1 ug/mL	N.D.
Complement C1q Sigma C1740 10 ug/mL	N.D.
Rat Tail Collagen Type I Sigma C7661 10 ug/mL	N.D.

N.D.: Not detectable

C. Precision

Intra-Assay Variation

Sample No.	Mean SAA-3 Levels (ng/mL)	Intra-Assay %CV
1	0.2	14 %
2	1.8	3%

Inter-Assay Variation

Sample No.	Mean SAA-3 Levels (ng/mL)	Inter-Assay %CV
1	0.2	13 %
2	1.8	10 %

The assay variations of Millipore Mouse SAA-3 ELISA kits were studied on two samples with varying concentrations of exogenous SAA-3. Intra-assay variation was calculated from eight determinations from a single assay. Inter-assay variation was calculated from single determinations in duplicate from seven separate assays.

XV. ASSAY CHARACTERISTICS (continued)

D. Spike and Recovery

Exogenous Mouse SAA-3	% Expected (n=5)
0.313 µg/mL	97.0± 30
0.625 µg/mL	96 ± 23
1.250 µg/mL	101 ± 19

Three serum and two plasma samples were spiked with different amounts of exogenous Mouse SAA-3. These spiked serum and plasma samples were assayed by Mouse SAA-3 ELISA. Expected values are the basal levels plus the spiked amount (0.313, 0.625and 1.25 µg/mL) of Mouse SAA-3. The % Expected is observed value divided by expected value X 100 (Mean ± SD).

XVI. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

XVII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.

2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practices.

3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started, all steps should be completed with precise timing and without interruption.

4. Avoid cross contamination of any reagents or samples to be used in the assay.

5. Make sure that all reagents and samples are added to the bottom of each well.

6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or well cross contamination due to inappropriate mixing.

7. Remove any air bubbles formed in the well after the addition of substrate because bubbles interfere with spectrophotometric readings.

8. Do not let the absorbency reading of the highest standard reach 2.0 units or higher before adding the stop solution.

9. High absorbance in background or blank wells could be due to 1.) well cross contamination by standard solution or sample and 2.) inadequate washing of wells with HRP.

XVIII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Mouse SAA-3 ELISA Plate	EP12
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Mouse SAA-3 ELISA Standard	E8012-K
Quality Controls 1 & 2	E6012-K
Assay Buffer (10 mL/vial)	EABTR
Enzyme Solution (12 mLl/vial)	EHRP
Mouse SAA-3 Detection Antibody (11 mL/vial)	E1012
Substrate (12mL)	ESS-TMB
Stop Solution (12 mL/vial)	ET-TMB

XIX. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (800) MILLIPORE
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to Canine or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.