HUMAN APOLIPOPROTEIN AIV (APO AIV) ELISA KIT

96-Well Plate (Cat. # EZHAP0A4-73K)

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I. INTENDED USE

This Human Apolipoprotein AIV (APO AIV) ELISA kit is used for the non-radioactive quantification of Human APO AIV in serum, plasma, and cell culture samples. This kit specifically measures Human APO AIV and has no cross reactivity to other Human apolipoproteins and Mouse or Rat APO AIV. One kit is sufficient to measure 38 unknown samples in duplicate.
This kit is for research purpose only.

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of Human APO AIV molecules from samples to the wells of a microtiter plate coated with a polyclonal anti-human APO AIV antibody, 2) washing of unbound materials from samples, 3) binding of a second biotinylated polyclonal anti-human APO AIV antibody to the captured molecules , 4) washing of unbound materials from samples, 5) binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies, 6) washing of excess 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 absorbance at 450 nm – 590 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured Human APO AIV 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 Human APO AIV.

III. REAGENTS SUPPLIED

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

A. Human Apo AIV ELISA Plate
Coated with polyclonal anti-Human APO AIV Antibody
Quantity: 1 strip plate
Preparation: Ready to Use
Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 ℃.

B. Adhesive Plate Sealer
Quantity: 2 sheets
Preparation: Ready to Use

C. 10X Concentrate HRP Wash Buffer
10X concentrate of 50 mM Tris Buffered Saline containing Tween-20
Quantity: 2 bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or deionized water

D. Human APO AIV Standard
Purified Recombinant Human APO AIV, lyophilized
Quantity: 0.5mL upon hydration
Preparation: Reconstitute with 0.5mL distilled or deionized water.

E. Quality Controls 1 and 2
One vial each, lyophilized, containing recombinant human APO AIV at two different levels.
Quantity: 0.5mL upon hydration
Preparation: Reconstitute each control with 0.5mL distilled or deionized water.

F. Assay Buffer (Sample Diluent)
0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 0.1 % BSA and 1.0 % Triton-X100
Quantity: 2 bottles containing 50 mL each
Preparation: Ready to Use

G. Assay Running Buffer
0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 0.1 % BSA, 1.0 % Triton-X100 and proprietary inhibitor
Quantity: 11 mL
Preparation: Ready to Use

H. Human APO AIV Detection Antibody
Pre-titered Biotinylated Rabbit anti-Human APO AIV Antibody
Quantity: 11 mL
Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

I. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL
Preparation: Ready to Use

J. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3', 5, 5'-tetramethylbenzidine in buffer
Quantity: 12 mL
Preparation: Ready to Use.

K. Stop Solution (Caution: Corrosive Solution)

0.3 M HCl
Quantity: 12 mL
Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze diluted HRP Wash Buffer, Assay Buffer, and reconstituted APO AIV Standards and Controls at ≤ –20°C. Minimize repeated freeze and thaw of the APO AIV 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. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

B. 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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 µL - 20 µL or 20 µL - 100 µL
2. Multi-Channel Pipettes and Pipette Tips: 5 ~ 50 µL and 50 ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at $4 \pm 2^{o}C$.

3. Transfer and store serum samples in separate tubes. Date and identify each sample.

4. Use freshly prepared serum or aliquot and store samples at $\leq -20^{o}C$ for later use. For long-term storage, keep at $-70\,^{o}C$. Avoid freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

6. If heparin is to be used as an anticoagulant, 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. SAMPLE PREPARATION

1. Allow all the reagents to come to room temperature.

2. Dilute serum or plasma samples 1:501 in Assay Buffer Sample Diluent (Cat # EABTR-1). Recommended Dilution: dilute 5 µl of serum or plasma in 2.5 mL of Assay Buffer Sample Diluent (Cat # EABTR-1). Cellular extract and culture media dilutions will vary.

IX. REAGENT PREPARATION

A. Standard and Quality Control Preparation

Human APO AIV Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human APO AIV Standard with 0.5 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 0.25 mL Assay Buffer Sample Diluent (EABTR-1) to each of the six tubes. Prepare serial dilutions by adding 0.25 mL of the reconstituted standard to Tube 1, mix well and transfer 0.25 mL of Tube 1 to Tube 2, mix well and transfer 0.25 mL of Tube 2 to Tube 3, mix well and transfer 0.25 mL of Tube 3 to Tube 4, mix well and transfer 0.25 mL of Tube 4 to Tube 5, mix well and transfer 0.25 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.

IX. REAGENT PREPARATION (continued)

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

Tube #	Volume of Assay Buffer Sample Diluent (EABTR-1) to Add	Volume of Standard to Add	Standard Concentration (ug/mL)
Tube 1	0.25 mL	0.25 mL of reconstituted standard	X/2
Tube 2	0.25 mL	0.25 mL of Tube 1	X/4
Tube 3	0.25 mL	0.25 mL of Tube 2	X/8
Tube 4	0.25 mL	0.25 mL of Tube 3	X/16
Tube 5	0.25 mL	0.25 mL of Tube 4	X/32
Tube 6	0.25 mL	0.25 mL of Tube 5	X/64

B. Human APO AIV Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Human APO AIV Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

X. ASSAY PROCEDURE

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

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized or distilled water. (Dilute both bottles with 900 mL deionized water).

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and wash each well 3 times with 300 µl of 1X HRP wash buffer per wash. 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 in duplicate 100 µL **Assay Running Buffer (EARB-5)** to Background wells.

4. Add in duplicate 80 µL **Assay Running Buffer (EARB-5)** to all Human APO AIV Standard, QC 1, and QC 2 and sample wells

5. Add in duplicate 20 µL Human APO AIV Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells. Add sequentially 20 µL of the diluted unknown samples in duplicate to the remaining wells. **For best result 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, approximately 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 firmly after each wash to remove residual buffer.

9. Add 100 µL of Detection Antibody to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 1 hour on the microtiter plate shaker.

10. Remove plate sealer and decant solutions 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.

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

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 to 20 minutes. Blue color should be formed in wells of APO AIV standards with intensity proportional to increasing concentrations of APO AIV.

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 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 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of highest APO AIV standard should be approximately 2.0-3.0, or not to exceed the capability of the plate reader used.

Assay Procedure for Human APO AIV ELISA Kit (Cat. # EZHAP0A4-73K)

	Step 1	Step 2	Step 3-4	Step 5	Step 6-8	Step 9	Step 10-11	Step 12	Step 13-14	Step 15	Step 15	Step 16	Step 16
Well #	**Dilute each bottle of 10X Wash Buffer with 450mL Deionized Water.**	**Wash plate 3X with 300 µl 1X Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels**	Assay Running Buffer	Standards/ Controls/ Samples	**Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer**	Detection Antibody	**Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µl Wash Buffer**	Enzyme Solution	**Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 6X with 300 µl Wash 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			80 µl	20 µl of Tube 6									
E1, F1			80 µl	20 µl of Tube 5									
G1, H1			80 µl	20 µl of Tube 4									
A2, B2			80 µl	20 µl of Tube 3									
C2, D2			80 µl	20 µl of Tube 2									
E2, F2			80 µl	20 µl of Tube 1									
G2, H2			80 µl	20 µl of reconstituted standard									
A3, B3			80 µl	20 µl of QC 1									
C3, D3			80 µl	20 µl of QC 2									
E3, F3			80 µl	20 µl of Sample									
G3, H3			80 µl	20 µl of Sample									
A4, B4 ↓			80 µl	20 µl of Sample									

XI. MICROTITER PLATE ARRANGEMENT

Human APO AIV ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3	QC 1	Etc.								
B	Blank	Tube 3	QC 1	Etc.								
C	Tube 6	Tube 2	QC2									
D	Tube 6	Tube 2	QC2									
E	Tube 5	Tube 1	Sample 1									
F	Tube 5	Tube 1	Sample 1									
G	Tube 4	Reconstituted Standard	Sample 2									
H	Tube4	Reconstituted Standard	Sample 2									

XII. CALCULATIONS

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. Final values are obtained by multiplying the calculated values by dilution factor of 501.

Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume deficit with assay buffer (sample diluent).

XIII. INTERPRETATION

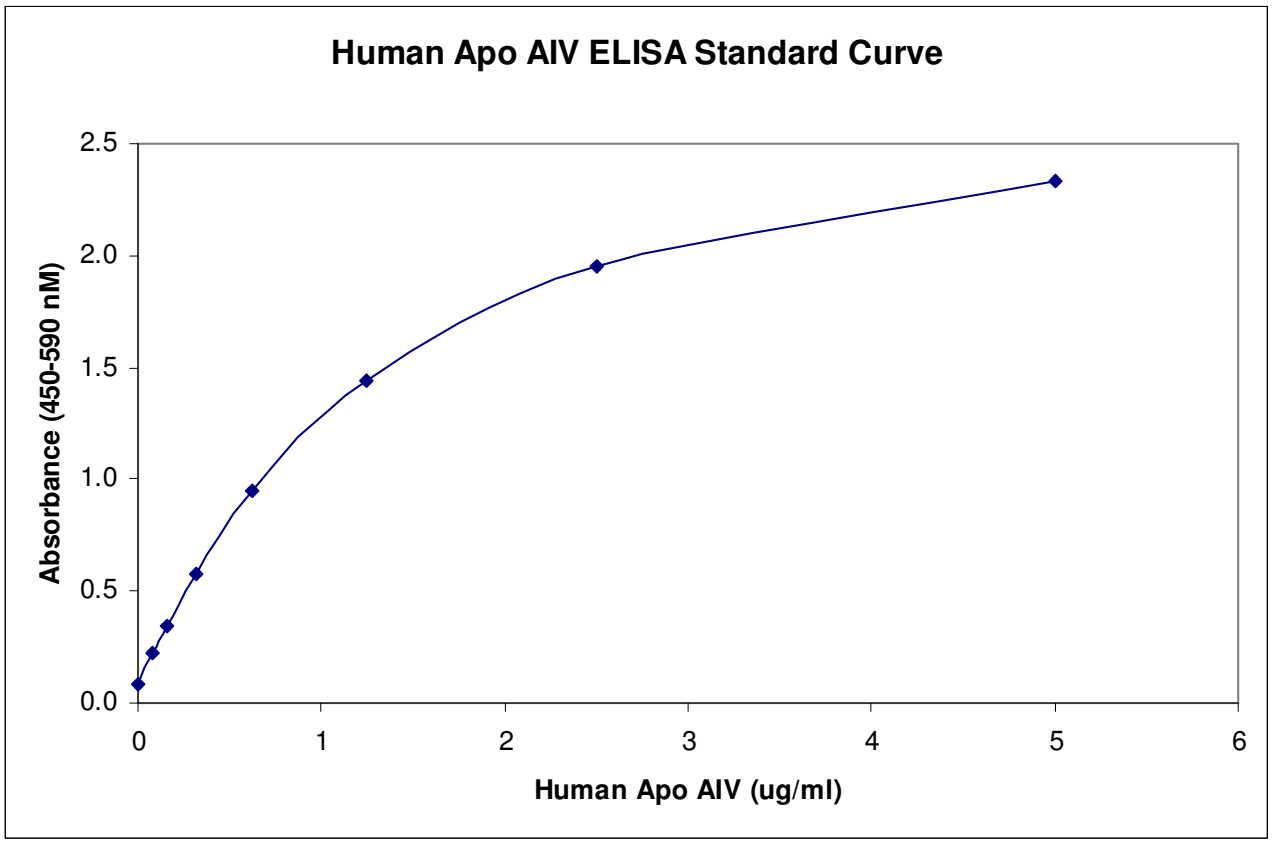
1. The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor.

2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.

3. The limit of sensitivity of this assay is 0.078 ug/mL Human APO AIV (20 µl sample size).

4. The appropriate range of this assay is 0.078 ug/mL to 5.0 ug/mL Human APO AIV (20 µl sample size). Any result greater than 5.0 ug/mL in a 20 µL of 1/501 diluted sample should be further diluted using Assay Buffer Sample Diluent (Cat # EABTR-1), and the assay repeated until the results fall within range.

XIV. STANDARD CURVE



Typical Standard Curve, not to be used to calculate data.

XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of APO AIV that can be detected by this assay is 0.078 ug/mL when using a 20 µL sample size.

B. Specificity

The antibody pair used in this assay is specific to Human APO AIV.
No species cross-reactivity is observed in the assay to rat, mouse, canine, porcine, or sheep samples. However, there is a high cross-reactivity to non-human primate samples that has not been evaluated or calibrated.

No cross reactivity is observed to the following human analytes:
Apo A1, Apo AII, Apo AV, Apo B, Apo CII, Apo CIII, Apo E, and other human cytokines and endocrine hormones tested.

C. Precision

Intra-Assay and Inter-Assay Variation

Sample no.	Mean µg/mL	Intra-Assay %CV	Inter-Assay %CV
1	0.11	3.5	11.3
2	1.06	5.7	13.2

Intra- and inter-assay variations were performed on two samples containing low and high concentrations of Human Apo AIV. Data (mean and %CV) shown are from one assay with eight duplicate determinations of each sample for intra-assay precision. For inter-assay precision, data are generated using six separate assays run for low and high Apo AIV concentrations in duplicate.

XV. ASSAY CHARACTERISTICS (continued)

D. Recovery

Sample Type	Human Serum (n=4) % Expected
0.31 ug/mL	102.9 ± 1.4
0.93 ug/mL	102.1 ± 4.1
2.78 ug/mL	91.3 ± 7.4

Human serum samples were spiked with different amounts of exogenous human Apo AIV and the assayed by Human Apo AIV ELISA. Expected values are the basal levels plus the spiked amount (0.312, 0.625, 1.25 and 2.5 ug/mL) of Human Apo AIV. The % Expected is observed value divided by expected value X 100 (Mean ± SD).

E. Linearity and Dilution

Sample Type Dilution Factor	Human Serum (n=4) % Expected
1/8	77.4 ± 4.1
1/4	92.1 ± 0.4
1/2	100.0 ± 0.0

Human serum samples (1/200 dilution) were diluted 1/2, 1/4 and 1/8 with Assay Buffer Sample Diluent and then assayed by Human Apo AIV ELISA. % Expected values (mean ± SD) are 1/2, 1/4 and 1/8 of the 20 uL sample (1/200 dilution) value.

XVI. QUALITY CONTROLS

The range for Quality Controls 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 practice.

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 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 cross well contamination due to inappropriate mixing.

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

8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.

9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XVIII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Microtiter Plate	EP73
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Human APO AIV Standard	E8073-K
Quality Controls 1 and 2	E6073-K
Assay Buffer Sample Diluent	EABTR-1
Assay Running Buffer	EARB-5
Human APO AIV Detection Antibody	E1073
Enzyme Solution	EHRP
Substrate	ESS-TMB
Stop Solution	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 (866) 441-8400
(636) 441-8400

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.

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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.