AESKULISA Ethanolamin-A

Instruction manual

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1. Intended Use

AESKULISA Ethanolamin-A is a solid phase enzyme immunoassay with highly purified phosphatidylethanolamin plus native human ß2-glycoprotein I for the quantitative and qualitative detection of IgA antibodies against phosphatidyl-ethanolamin in human serum. These antibodies recognize specific epitopes on a complex composed out of phosphatidyl-ethanolamin and ß2-glycoprotein I.

The assay is an aid in the diagnosis and risk estimation of thrombosis in patients with systemic lupus erythematosus and APS.

2. Clinical Application and Principle of the Assay

Antibodies against phosphatidyl-ethanolamin, an phospholipid derived from glycerol, belong to the group of anti-phospholipid antibodies specific for phospholipids such as cardiolipin, phosphatidyl-serine, -inositol, -choline, sphingomyelin and phosphatidic acid. Phospholipids are components of biological membranes.

Anti-phospholipid antibodies are frequently found in sera of patients with systemic lupus erythematosus (SLE) and related diseases. The occurrence of anti-phospholipid antibodies in patients with SLE and related diseases is typical for a secondary anti-phospholipid syndrome (APS). In contrast, anti-phospholipid antibodies in patients with no other autoimmune diseases characterize the primary APS.

Many studies have shown a correlation between these autoantibodies and an enhanced incidence of thrombosis, thrombocytopenia and habitual abortions (as a consequence of placental infarction). The exact mechanisms by which pathogenic anti-phospholipid antibodies induce thrombosis is not yet revealed fully.

Reports of anti-phosphatidyl-ethanolamin antibodies with similar or identical pathogenic associations as those described for anti-cardiolipin and anti-phosphatidylserine have repeatedly been described in the literature. PE is a zwitterionic phospholipid present in both internal and external sides of cell mebranes, thought to be involved in the development of thrombotic events via its role in the protein C pathway and the inactivation of factor Va by activated protein C. Antibodies directed to PE could play an important role in the pathogenesis of the APS by inhibiting activated protein C. In some instances, anti-PE antibodies are the sole antiphospholipid antibodies observed thus being an important diagnostic tool in addition to cardiolipin antibodies.

Principle of the test

Serum samples diluted 1:101 are incubated in the microplates coated with the specific antigen. Patient's antibodies, if present in the specimen, bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off in the following step. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The rate of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample.

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3. Kit Contents

To be reconstituted:

5x Sample Buffer 1 vial, 20 ml - 5x concentrated (capped white: yellow solution)

Containing: Tris, NaCl, BSA, sodium azide < 0.1% (preservative)

50x Wash Buffer 1 vial, 20 ml - 50x concentrated (capped white: green solution)

Containing: Tris, NaCl, Tween 20, sodium azide < 0.1% (preservative)

Ready to use:

Negative Control 1 vial, 1.5 ml (capped green: colorless solution)

Containing: Human serum (diluted), sodium azide < 0.1% (preservative)

Positive Control 1 vial, 1.5 ml (capped red: yellow solution)

Containing: Human serum (diluted), sodium azide < 0.1% (preservative)

Cut-off Calibrator 1 vial, 1.5 ml (capped blue: yellow solution)

Containing: Human serum (diluted), sodium azide < 0.1% (preservative)

Calibrators 6 vials, 1.5 ml each 0, 3, 10, 30, 100, 300 U/ml

(color increasing with concentration: yellow solutions)

Containing: Human serum (diluted), sodium azide < 0.1% (preservative)

Conjugates 1 vial,15 ml IgA (capped red: red solution)

Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase

TMB Substrate 1 vial, 15 ml (capped black)

Containing: Stabilized TMB/H2O2

Stop Solution 1 vial, 15 ml (capped white: colorless solution)

Containing: 1M Hydrochloric Acid

Microtiterplate 12x8 well strips with breakaway microwells

Coating see paragraph 1

Material required but not provided:

Microtiter plate reader 450 nm reading filter and optional 620 nm reference filter (600-690 nm). Glass ware(cylinder 100-1000ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 μ l) or adjustable multipipette (100-1000ml). Microplate washing device (300 μ l repeating or multichannel pipette or automated system), adsorbent paper.

Our tests are designed to be used with purified water according to the definition of the United States Pharmacopeia (USP 26 - NF 21) and the European Pharmacopeia (Eur.Ph. 4th ed.).

4. Storage and Shelf Life

Store all reagents and the microplate at 2-8°C/35-46°F, in their original containers. Once prepared, reconstituted solutions are stable for 1 month at 4°C/39°F, at least. Reagents and the microplate shall be used within the expiry date indicated on each component, only. Avoid intense exposure of TMB solution to light. Store microplates in designated foil, including the desiccant, and seal tightly.

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5. Precautions of Use

5.1 Health hazard data

This product is for **IN VITRO DIAGNOSTIC USE** only. Thus, only staff trained and specially advised in methods of in vitro diagnostics may perform the kit. Although this product is not considered particularly toxic or dangerous in conditions of normal use, refer to the following for maximum safety:

Recommendations and precautions

This kit contains potentially hazardous components. Though kit reagents are not classified being irritant to eyes and skin we recommend to avoid contact with eyes and skin and wear disposable gloves.

WARNING! Calibrators, Controls and Buffers contain sodium azide (NaN₃) as a preservative. NaN₃ may be toxic if ingested or adsorbed by skin or eyes. NaN₃ 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. Please refer to decontamination procedures as outlined by CDC or other local/national guidelines.

Do not smoke, eat or drink when manipulating the kit.

Do not pipette by mouth.

All human source material used for some reagents of this kit (controls, standards e.g.) has been tested by approved methods and found negative for HbsAg, Hepatitis C and HIV 1. However, no test can guarantee the absence of viral agents in such material completely. Thus handle kit controls, standards and patient samples as if capable of transmitting infectious diseases and according to national requirements.

5.2 General directions for use

Do not mix or substitute reagents or microplates from different lot numbers. This may lead to variations in the results.

Allow all components to reach room temperature (20-32°C/68-89.6°F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Incubation: We recommend test performance at 30°C/86°F for automated systems.

Never expose components to higher temperature than 37°C/98.6 °F.

Always pipette substrate solution with brand new tips only. Protect this reagent from light. Never pipette conjugate with tips used with other reagents prior.

A definite clinical diagnosis should not be based on the results of the performed test only, but should be made by the physician after all clinical and laboratory findings have been evaluated. The diagnosis is to be verified using different diagnostic methods.

6. Sample Collection, Handling and Storage

Use preferentially freshly collected serum samples. Blood withdrawal must follow national requirements.

Do not use icteric, lipemic, hemolysed or bacterially contaminated samples. Sera with particles should be cleared by low speed centrifugation ($<1000 \times g$). Blood samples should be collected in clean, dry and empty tubes. After separation, the serum samples should be used immediately, respectively stored tightly closed at $2-8^{\circ}$ C/ $35-46^{\circ}$ F up to three days, or frozen at -20° C/ -4° F for longer periods.

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7. Assay Procedure

7.1 Preparations prior to pipetting

Dilute concentrated reagents:

Dilute the concentrated sample buffer 1:5 with distilled water (e.g. 20 ml plus 80 ml). Dilute the concentrated wash buffer 1:50 with distilled water (e.g. 20 ml plus 980 ml).

Samples:

Dilute serum samples 1:101 with sample buffer (1x) e.g. 1000 μl sample buffer (1x) + 10 μl serum. Mix well!

Washing:

Prepare 20 ml of diluted wash buffer (1x) per 8 wells or 200 ml for 96 wells e.g. 4 ml concentrate plus 196 ml distilled water.

Automated washing:

Consider excess volumes required for setting up the instrument and dead volume of robot pipette.

Manual washing:

Discard liquid from wells by inverting the plate. Knock the microwell frame with wells downside vigorously on clean adsorbent paper. Pipette 300 µl of diluted wash buffer into each well, wait for 20 seconds. Repeat the whole procedure twice again.

Microplates:

Calculate the number of wells required for the test. Remove unused wells from the frame, replace and store in the provided plastic bag, together with desiccant, seal tightly (2-8°C/35-46°F).

7.2 Work flow

For pipetting scheme see Annex A, for the test procedure see Annex B We recommend pipetting samples and calibrators in duplicate.

Cut-off calibrator should be used for qualitative testing only.

- Pipette 100 μl of each patient's diluted serum into the designated microwells.
- Pipette 100 µl calibrators OR cut-off calibrator and negative and positive controls into the designated wells.
- Incubate for 30 minutes at 20-32°C/68-89.6°F.
- Wash 3x with 300 μl washing buffer (diluted 1:50).
- Pipette 100 µl conjugate into each well.
- Incubate for 30 minutes at 20-32°C/68-89.6°F.
- Wash 3x with 300 µl washing buffer (diluted 1:50).
- Pipette 100 µl TMB substrate into each well.
- Incubate for 30 minutes at 20-32°C/68-89.6°F, protected from intense light.
- Pipette 100 µl stop solution into each well, using the same order as pipetting the substrate.
- Incubate 5 minutes minimum.
- Agitate plate carefully for 5 sec.
- Read absorbance at 450 nm (optionally 450/620 nm) within 30 minutes.

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8. Quantitative and Qualitative Interpretation

For **quantitative interpretation** establish the standard curve by plotting the **optical density (O.D.) of each calibrator (y-axis)** with respect to the corresponding concentration values in **U/ml (x-axis)**. For best results we recommend log/lin coordinates and 4-Parameter Fit. From the O.D. of each sample, read the corresponding antibody concentrations expressed in **U/ml**.

Normal Range	Equivocal Range	Positive Results
< 12 U/ml	12 - 18 U/ml	>18 U/ml

Example of a standard curve

We recommend pipetting calibrators in parallel for each run.

Calibrators IgA	OD 450/620 nm	CV % (Variation)
0 U/ml	0.053	6.9
3 U/ml	0.172	3.3
10 U/ml	0.330	1.3
30 U/ml	0.663	1.9
100 U/ml	1.302	1.0
300 U/ml	2.115	0.0

Example of calculation

Patient	Replicate (OD)	Mean (OD)	Result (U/ml)
P 01	0.863/0.852	0.858	51.0
P 02	0.536/0.576	0.556	24.8

For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an inhouse Quality Control by using own controls and/or internal pooled sera, as foreseen by EU regulations.

Do not use this example for interpreting patients results!

Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures.

For qualitative interpretation read the optical density of the cut-off calibrator and the patient samples. Compare patient'sOD with the OD of the cut-off calibrator. For qualitative interpretation we recommend to consider sera within a range of 20% around the cut-off value as equivocal. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

Negative: OD patient < 0.8 x OD cut-off

Equivocal: $0.8 \times OD_{cut-off} \le OD_{patient} \le 1.2 \times OD_{cut-off}$

Positive OD patient > 1.2 x OD cut-off

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9. Technical Data

Sample material: serum

Sample volume: 10 μl of sample diluted 1:101 with 1x sample buffer

Total incubation time: 90 minutes at 20-32°C/68-89.6°F

Calibration range: 0-300 U/ml

Analytical sensitivity: 1.0 U/ml

Storage: at 2-8°C/35-46°F use original vials, only

Number of determinations: 96 tests

10. Performance Data

10.1 Analytical sensitivity

Testing sample buffer 30 times on *AESKULISA Ethanolamin-A* gave an analytical sensivity of 1.0 U/ml.

10.2 Specificity

The microplate is coated with **phosphatidyl-ethanolamin and native human ß2-glycoprotein I.** No crossreactivities to other autoantigens have been found.

10.3 Linearity

Chosen sera have been tested with this kit and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be samples that do not follow this rule.

		measured	expected	
Sample	Dilution	concentration	concentration	Recovery
No.	Factor	(U/ml)	(U/ml)	(%)
1	1 / 100	234.0	240.0	97.5
	1 / 200	115.0	120.0	98.8
	1 / 400	56.9	60.0	94.8
	1 / 800	27.9	30.0	93.0
2	1 / 100	61.2	58.4	104.8
	1 / 200	29.0	29.2	99.3
	1 / 400	13.9	14.6	95.2
	1 / 800	6.9	7.3	94.5

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10.4 Precision

To determine the precision of the assay, the variability (intra and inter-assay) was assessed by examining its reproducibility on three serum samples selected to represent a range over the standard curve.

In	tra-Assa	ay
Sample	Mean	CV
No.	(U/ml)	(%)
1	238.0	3.6
2	58.0	6.9
3	23.0	5.3

In	ter-Assa	ay
Sample	Mean	CV
No.	(U/ml)	(%)
1	241.0	3.1
2	60.0	5.8
3	20.0	5.9

10.5 Calibration

Due to the lack of international reference calibration this assay is calibrated in arbitrary units (U/ml).

11. Literature

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ANNEX A: Pipetting scheme

We suggest pipetting calibrators, controls and samples as follows:

For quantitative interpretation use calibrators to establish a standard curve.

For qualitative interpretation use cut-off calibrator.

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	1	2	3	4	5	6	7	8	9	10	11	12
Α	CalA	CalE	P1				NC	P2				
В	CalA	CalE	P1				NC	P2				
С	CalB	CalF	P2				CC	P3				
D	CalB	CalF	P2				CC	P3				
Ε	CalC	PC	P3				PC					
F	CalC	PC	P3				PC					
G	CalD	NC					P1					
Н	CalD	NC					P1					

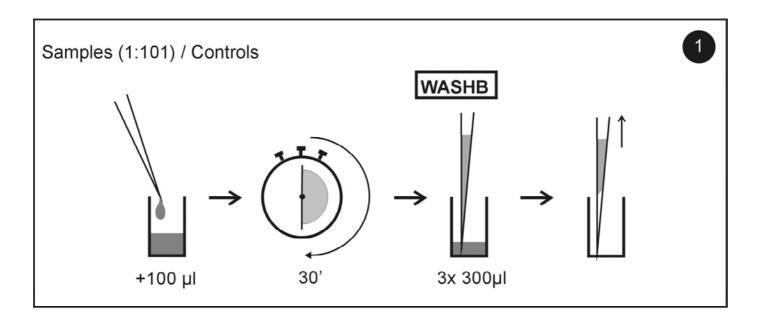
CalA: calibrator A, CalB: calibrator B, CalC: calibrator C, CalD: calibrator D, CalE: calibrator E,

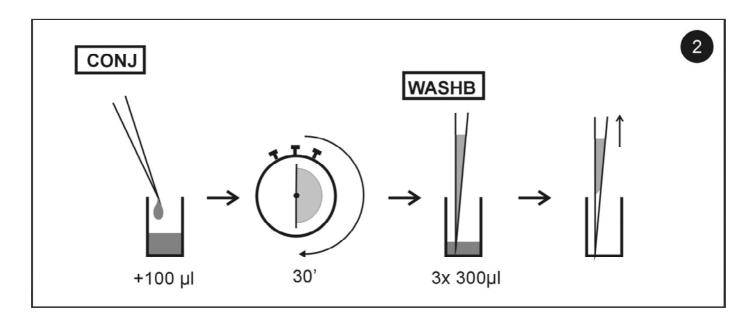
CalF: calibrator F PC: positive control NC: negative control CC: Cut-off calibrator

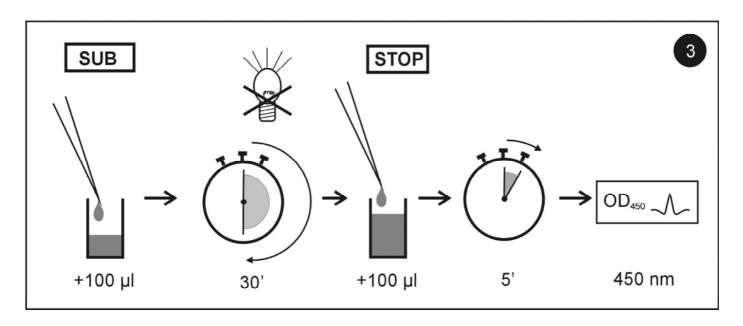
P1: patient 1 P2: patient 2 P3: patient 3

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Annex B: Test Procedure







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	COCAL	◆ Etalon Seuil	 Calibrador de cut-off
	CO-CAL	 Grenzwert Kalibrator 	 Οριακός ορός Αντιδραστήριο βαθμονόμησ
◆ Calibrador de cut-off		◆ Calibrador de cut-off	
◆ Controllo positivo ◆ Positive Control		◆ Controllo positivo	◆ Positive Control
↑ Contrôle Positif	CONI+	◆ Contrôle Positif	◆ Control Positivo
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◆ Controlo positivo		◆ Controlo positivo	
◆ Controllo negativo ◆ Negative Control		◆ Controllo negativo	◆ Negative Control
◆ Control Negatir	CONI		<u> </u>
♦ Negativ Kontrolle ♦ Αρνητικός ορός ελέγχου	CON_	=	 Αρνητικός ορός ελέγχου
◆ Controlo negativo		·	
◆ Calibrator			▲ Calibrator
CAL ♦ Etalon ♦ Calibrador ♦ Ayriδραστήριο βαθμονόμησης	CAI		
	I UAL I		◆ Calibrador
		♦ Kalibrator	◆ Calibrador
♦ Recupero • Recovery		◆ Kalibrator◆ Calibrador	◆ Calibrador◆ Αντιδραστήριο βαθμονόμησης
I RU. II			 Calibrador Αντιδραστήριο βαθμονόμησης Recovery
▼ Wiedernindung ▼ Avaktijori	[RC]	★ Kalibrator ★ Calibrador ★ Recupero ★ Corrélation	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado
·	RC	 ★ Kalibrator ★ Calibrador ★ Recupero ★ Corrélation ★ Wiederfindung 	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery
	RC	 ★ Kalibrator ★ Calibrador ★ Recupero ★ Corrélation ★ Wiederfindung ★ Recuperacão 	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση
CON.I		 ★ Kalibrator ★ Calibrador ★ Recupero ★ Corrélation ★ Wiederfindung ✦ Recuperacão ✦ Coniugato 	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση Conjugate
V ποιηugat V Σοςεογρα		Kalibrator Calibrador Recupero Corrélation Wiederfindung Recuperacão Coniugato Conjugé	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση Conjugate Conjugado
	RC	Kalibrator Calibrador Recupero Corrélation Wiederfindung Recuperação Coniugato Conjugé Konjugat	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση Conjugate
		Kalibrator Calibrador Recupero Corrélation Wiederfindung Recuperação Coniugato Conjugé Konjugat Conjugado	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση Conjugate Conjugado Σύζευγμα
	CONJ	Kalibrator Calibrador Recupero Correlation Widederfindung Recuperacão Coniugato Conjugé Konjugat Conjugado Micropiastra rivestita	 Calibrador Αντιδραστήριο βαθμονόμησης Recovery Recuperado Ανάκτηση Conjugate Conjugado Σύζευγμα Coated microtiter plate
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