AESKULISA ß2-Glyco-A

Protocol 30-30-30 REF30-7205US

Instruction manual

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1. Intended Use

AESKULISA ***β2 Glyco-A*** is a solid phase enzyme immunoassay employing native β2 glycoprotein I highly purified from human plasma for the semiquantitative and qualitative detection of IgA antibodies against β2 glycoprotein I in human serum.
The presence of anti-β2 glycoprotein I antibodies in conjunction with clinical findings and other laboratory results can be used as an aid in the diagnosis of thrombotic disorders related to primary and secondary antiphospholipid syndrome.

2. Clinical Application and Principle of the Assay

Antibodies against β2-glycoprotein I (β2-GPI) belong to the group of anti-phospholipid antibodies mainly targeting complexes composed of negatively charged phospholipids (e.g. cardiolipin) and plasma proteins like β2- glycoprotein I, prothrombin, protein C or protein S. Anti-phospholipid antibodies are serological markers of the antiphospholipid syndrome (APS), a systemic autoimmune disease characterized by venous and arterial thromboses and recurrent abortions. In contrast to primary APS that is unrelated to an underlying disease, secondary APS is associated with other autoimmune diseases, mainly with systemic lupus erythematosus (SLE).[1,2,3]
In addition to antibodies against the Cardiolipin/β2-GPI complex, reactivity against isolated β2- glycoprotein I is found, too. Thus β2-glycoprotein I is discussed to be an autoantigen on its own. The importance of β2-GPI antibodies in the diagnosis of APS has been emphasized by including them into the laboratory parameters of classification criteria for APS, which until recently (end 2005) contained Cardiolipin antibodies and lupus anticoagulant only.[4,5,6]
β2-glycoprotein I, also called apolipoprotein H, is a 50 kDa β2 globuline which is associated in vivo with lipoproteins, platelets and phospholipids and which seems to inhibit the intrinsic coagulation pathway, the prothrombinase activity and the ADP-dependent platelet aggregation.[7,8]
The exact mechanisms how pathogenic anti-phospholipid antibodies induce thrombosis has not been revealed yet.

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.

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, sodium azide < 0.1% (preservative)

Ready to use:

Negative Control 1 vial, 1.5 ml (capped green: yellow solution)
Containing: PBS, BSA, Human serum (diluted), sodium azide < 0,1% (preservative)

Positive Control 1 vial, 1.5 ml (capped red: yellow solution)
Containing: PBS, BSA, Human serum (diluted), sodium azide < 0,1% (preservative)

Cut-off Control 1 vial, 1.5 ml (capped blue: yellow solution)
Containing: PBS, BSA, 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: PBS, BSA, Human serum (diluted), sodium azide < 0,1% (preservative)

Conjugate 1 vial,15 ml IgA (capped red: red solution)
Containing: PBS, BSA, goat anti-human immunoglobulins conjugated to horseradish peroxidase (preservative)

TMB Substrate 1 vial, 15 ml (capped black)
Containing: Stabilized TMB/H_2O_2

Stop Solution 1 vial, 15 ml (capped white: colorless solution)
Containing: 1M Hydrochloric Acid

Microtiterplate 12x8 well strips with breakaway microwells
Coating highly purified native human β2 glycoprotein I

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 µl) or adjustable multipipette (100-1000µl). Microplate washing device (300 µl repeating or multi-channel 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.***

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 (NaN_3) as a preservative . NaN_3 may be toxic if ingested or adsorbed by skin or eyes. NaN_3 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 FDA 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 (20-26°C/64-78.8°F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

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.

Limitations

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 x 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°C/35-46°F up to three days, or frozen at -20°C/-4°F for longer periods.

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.

- Pipette 100 µl of each patient‘s diluted serum into the designated microwells.
- Pipette 100 µl calibrators OR cut-off control and negative and positive controls into the designated wells.
- Incubate for 30 minutes at room temperature (20-26°C/64-78.8°F).
- Wash 3x with 300 µl washing buffer (diluted 1:50).
- Pipette 100 µl conjugate into each well.
- Incubate for 30 minutes at room temperature (20-26°C/64-78.8°F).
- Wash 3x with 300 µl washing buffer (diluted 1:50).
- Pipette 100 µl TMB substrate into each well.
- Incubate for 30 minutes at room temperature (20-26°C/64-78.8°F).
- 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.

8. Semiquantitative and Qualitative Interpretation

For **semiquantitative interpretation** establish the standard curve by plotting the **optical density (OD) 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 OD of each sample, read the corresponding antibody concentrations expressed in **U/ml.**

Normal Range	Positive Results
≤ 15 U/ml	**> 15 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	0.041	1.7
3 U/ml	0.132	0.0
10 U/ml	0.280	2.6
30 U/ml	0.584	2.1
100 U/ml	1.211	0.0
300 U/ml	2.042	0.6

Example of calculation

Patient	Replicate (OD)	Mean (OD)	Result (U/ml)
P 01	0.756/0.757	0.757	45.9
P 02	1.344/1.352	1.348	123.3

For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an in-house Quality Control by using own controls and/or internal pooled sera, as foreseen by 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 control and the patient samples. Compare patient´s OD with the OD of the cut-off control. All samples which are higher than cut-off are considered positive.

Negative:	**OD patient < OD $_{\text{cut-off}}$**
Positive:	**OD patient > OD $_{\text{cut-off}}$**

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 room temperature (20-26°C/64-78.8°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 ß-2 Glyco A (REF7205US) gave an analytical sensitivity of 1.0 U/ml.

10.2 Specificity and sensitivity

The microplate is coated with highly purified native human ß2-glycoprotein **I.** 115 sera of patients suffering from APS, SLE and other autoimmune diseases have been tested on the AESKULISA ß-2 Glyco A and a predicate device (all tests were perfomed on the 30-15-15 protocol (REF7205US))

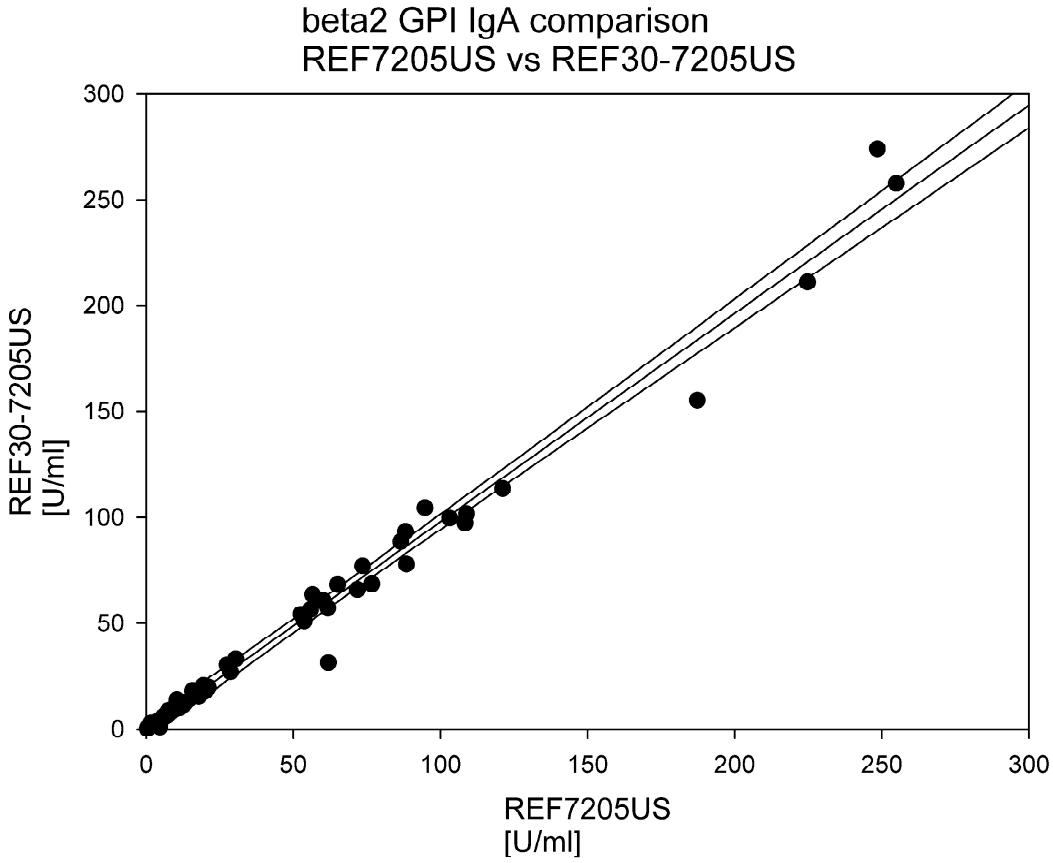
		predicate device		
		positive	negative	Total
AESKULISA ß-2 Glyco A	positive	17	7	24
	negative	8	83	93
		25	90	115

positive percent agreement 68.0% (17/25)
negative percent agreement 92.2% (83/90)
overall percent agreement 87.0% (100/115)

Disease	# Tested	# positive AESKU (%)	# positive pred dev (%)
Antiphospholipid-Syndrome	39	8 (20.5%)	11 (28.2%)
Systemic-Lupus-Erythematosus	46	7 (15.2%)	5 (10.9%)
SLE with secondary APS	17	9 (52.9%)	9 (52.9%)
suspected APS	1	0 (0%)	0 (0%)
indeterminate connective tissue disease with APS	1	0 (0%)	0 (0%)
healthy donors	11	0 (0%)	0 (0%)

The data has been aquired with the AESKULISA ß-2 Glyco A (REF7205US). The comparability of these data was assessed with 5 sera tested on both, REF 7205US (30-15-15 minute protocol) and REF 30-7205US (30-30-30 protocol). A linear regression analysis of the two products showed that the two protocols are equivalent. Included in these sera are 26 sera close to the cut-off (<30 U/ml).

Y = b[0] + b[1]X	value	range (CI95%)
b[0]	-0.47	-5.72 / 4.78
b[1]	0.984	0.940 / 1.028
r^2	0.976	



10.3 Linearity

Chosen sera have been tested with this kit and found to dilute linearly. However, due to the hetero- geneous nature of human autoantibodies there might be samples that do not follow this rule. (all tests were perfomed on the 30-15-15 protocol (REF7205US))

Sample No.	Dilution Factor	measured concentration (U/ml)	expected concentration (U/ml)	Recovery (%) 90 - 110 %
1	1 / 100	123.7	122.0	101.4
	1 / 200	63.1	61.0	103.4
	1 / 400	29.3	30.5	96.1
	1 / 800	14.7	15.3	96.1
2	1 / 100	126.2	118.0	107.0
	1 / 200	61.1	59.0	103.6
	1 / 400	27.9	29.5	94.6
	1 / 800	13.9	14.8	93.9

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. The data has been aquired with the 30-15-15 protocol (REF 7205US). The accepted range for CV is 10%. (n=24 / 18)

Intra-Assay			Inter-Assay		
Sample No.	Mean (U/ml)	CV (%)	Sample No.	Mean (U/ml)	CV (%)
1	13.0	7.6	1	14.4	8.4
2	96.5	8.0	2	106.7	9.6
3	118.2	8.7	3	124.7	8.6

10.5 Calibration

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

11. Literature

1. **Schousboe I. (1985)**:
   β*2-glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway.*
   Blood 66: 1086-1091.
2. **Nimpf J, Bevers EM, Bomans PH, Till U, Wurm H, Kostner GM, Zwaal RF et al. (1986)**:
   Prothrombinase activity of human platelets is inhibited by β*2-glycoprotein I.*
   Biochim Biophys Acta 884: 142-149.
3. **Nimpf J, Wurm H, Kostner GM. (1987):**
   β*2-glycoprotein I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation.*
   Artheriosclerosis 6: 109-114.
4. **Tincani A, Allegri F, Balestrieri G, Reber G, Sanmarco M, Meroni P, and Boffa MC(2004).**
   Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies.
   Thromb.Res. 114(5-6): 553-558.
5. **Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, DE Groot PG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, and Krilis SA(2006).**
   International consensus statement on an update of the classification criteria for definite anti phospholipid syndrome (APS).
   J.Thromb.Haemost. 4(2): 295-306.
6. **Wöhrle R, Matthias T, von Landenberg P, Oppermann M, Helmke K, Förger F (2000):**
   Clinical relecance of antibodies against different phospholipids.
   Journal of Autoimmunity 15: A60.
7. **Harris EN, Gharavi AE, Boey ML, et al. (1983):**
   Anticardiolipin antibodies: Detection by radioimmunassay and association with thrombosis in systemic lupus erythematosus.
   Lancet Nov 26: 1211-1214.
8. **Galli M, Comfurius P, Maassen C, et al. (1990):**
   Anticardiolipin antibodies directed not to cardiolipin but to a plasma protein cofactor.
   Lancet 335: 1544-1547.

ANNEX A: Pipetting scheme

We suggest pipetting calibrators, controls and samples as follows:

For **semi-quantitative interpretation** use calibrators to establisch a standart curve

For **qualitative interpretation** use cut-off control

For **semi-quantitative interpretation** use calibrators to establish a standard curve

	1	2	3	4	5	
A	CalA	CalE	P1			
B	CalA	CalE	P1			
C	CalB	CalF	P2			
D	CalB	CalF	P2			
E	CalC	PC	P3			
F	CalC	PC	P3			
G	CalD	NC	...			
H	CalD	NC	...			

For **qualitative interpretation** use cut-off control

	1	2	3	4	5	
A	NC	P2				
B	NC	P2				
C	CC	P3				
D	CC	P3				
E	PC	...				
F	PC	...				
G	P1	...				
H	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

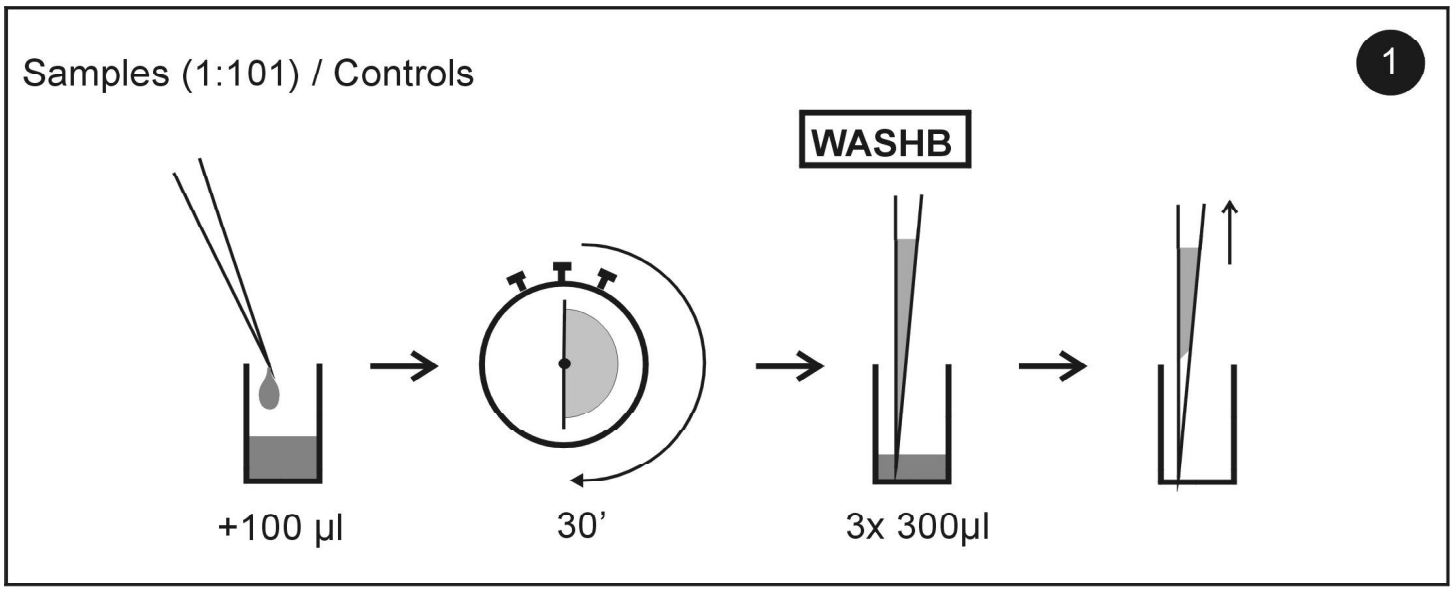
CC: Cut-off control

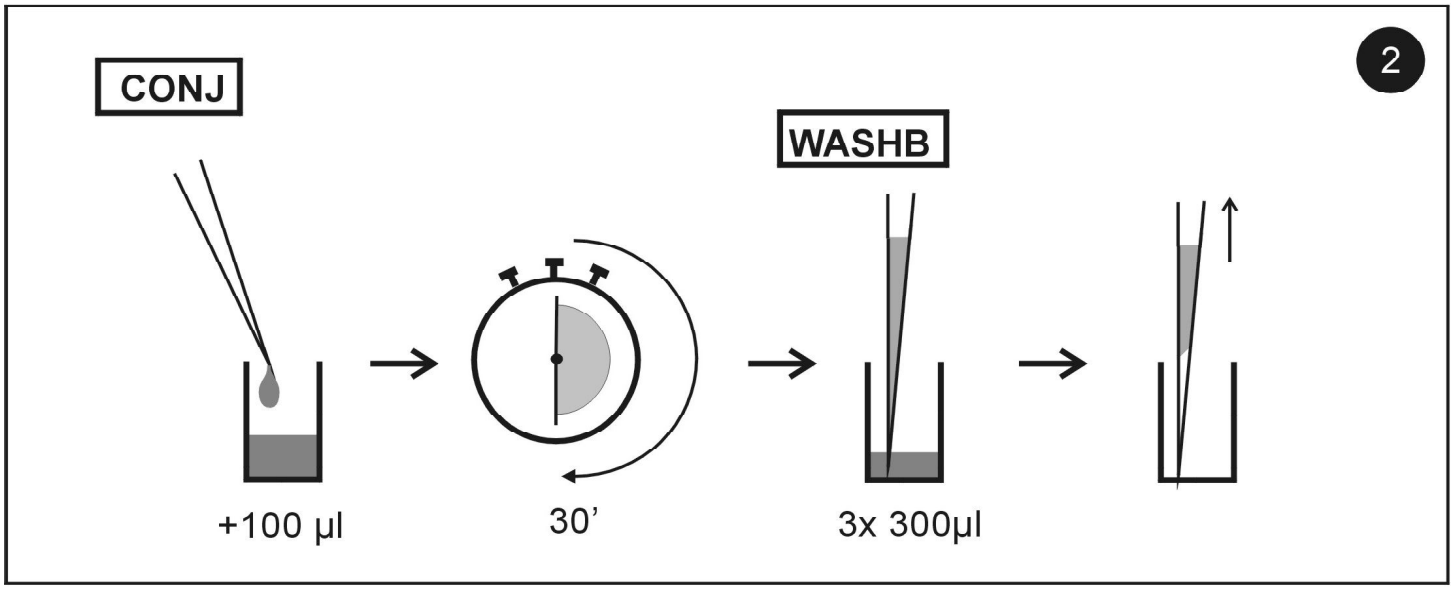
P1: patient 1

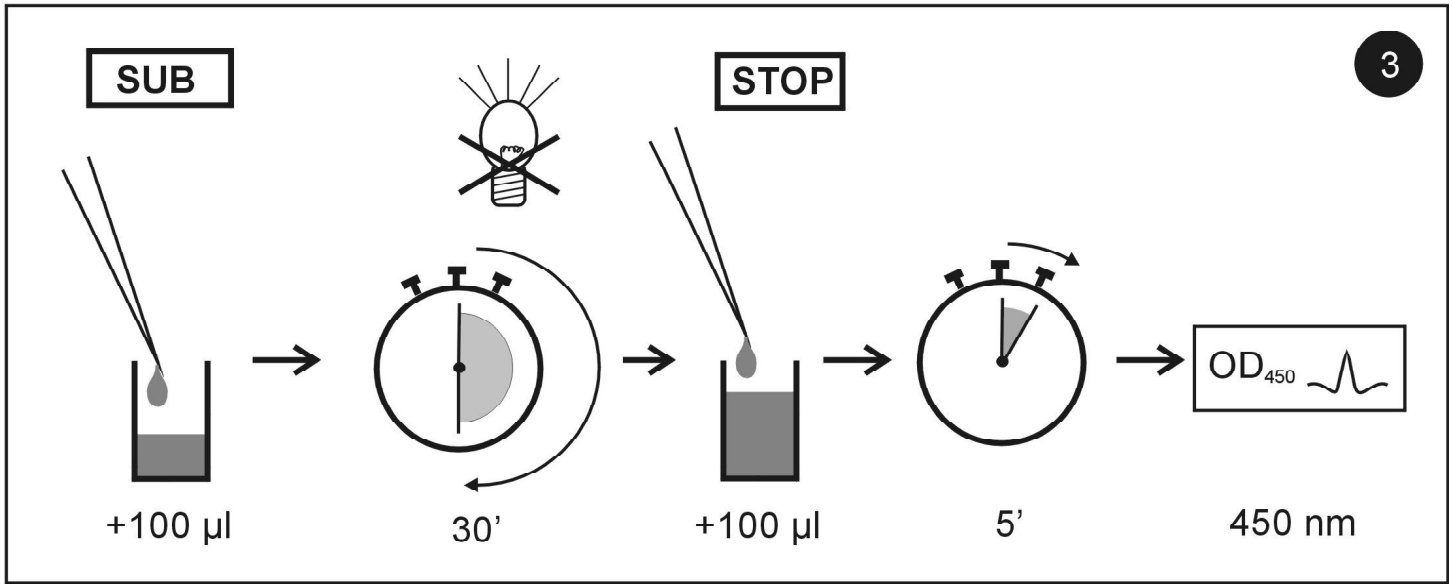
P2: patient 2

P3: patient 3

Annex B: Test Procedure/ Testablauf







Assay/Test: _____ Incubation / Inkub. : 1._____min Date/ Datum:_____

Temperature/Temperatur:_____ °F _____ °C 2._____min

Signature/Unterschrift:._____

Name:_____ 3._____min

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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IVD	♦ Diagnosi in vitro ♦ Pour diagnostic in vitro ♦ In Vitro Diagnostikum ♦ Para uso Diagnóstico in vitro	♦ For in vitro diagnostic use ♦ Para uso diagnóstico in vitro ♦ In Vitro Διαγνωστικό μέσο
REF	♦ Numero d'ordine ♦ Référence Catalogue ♦ Bestellnummer ♦ Número de catálogo	♦ Cataloge number ♦ Numéro de catálogo ♦ Αριθμός παραγγελίας
LOT	♦ Descrizione lotto ♦ Lot ♦ Chargen Bezeichnung ♦ Lote	♦ Lot ♦ Lote ♦ Χαρακτηρισμός παρτίδας
CE	♦ Conformità europea ♦ Déclaration CE de Conformité ♦ Europäische Konformität ♦ Déclaracão CE de Conformidade	♦ EC Declaration of Conformity ♦ Declaración CE de Conformidad ♦ Ευρωπαϊκή συμφωνία
96	♦ 96 determinazioni ♦ 96 tests ♦ 96 Bestimmungen ♦ 96 Testes	♦ 96 tests ♦ 96 pruebas ♦ 96 προσδιορισμοί
i	♦ Rispettare le istruzioni per l'uso ♦ Voir les instructions d'utilisation ♦ Gebrauchsanweisung beachten ♦ Ver as instrucões de uso	♦ See instructions for use ♦ Ver las instrucciones de uso ♦ Λάβετε υπόψη τις οδηγίες χρήσης
	♦ Da utilizzarsi entro ♦ Utilise avant le ♦ Verwendbar bis ♦ Utilizar antes de	♦ Use by ♦ Utilizar antes de ♦ Χρήση μέχρι
+2°C +8°C	♦ Conservare a 2-8°C ♦ Conserver à 2-8°C ♦ Lagerung bei 2-8°C ♦ Conservar entre 2-8°C	♦ Store at 2-8°C (35-46°F) ♦ Conservar a 2-8°C ♦ Φυλάσσεται στους 2-8°C
	♦ Prodotto da ♦ Fabriqué par ♦ Hergestellt von ♦ Fabricado por	♦ Manufactured by ♦ Fabricado por ♦ Κατασκευάζεται από
CO-CAL	♦ Calibratore cut-off ♦ Etalon Seuil ♦ Grenzwert Kalibrator ♦ Calibrador de cut-off	♦ Cut off Calibrator ♦ Calibrador de cut-off ♦ Οριακός ορός Αντιδραστήριο βαθμονόμησης
CON +	♦ Controllo positivo ♦ Contrôle Positif ♦ Positiv Kontrolle ♦ Controlo positivo	♦ Positive Control ♦ Control Positivo ♦ Θετικός ορός ελέγχου
CON −	♦ Controllo negativo ♦ Contrôle Négatif ♦ Negativ Kontrolle ♦ Controlo negativo	♦ Negative Control ♦ Control Negativo ♦ Αρνητικός ορός ελέγχου
CAL	♦ Calibratore ♦ Etalon ♦ Kalibrator ♦ Calibrador	♦ Calibrator ♦ Calibrador ♦ Αντιδραστήριο βαθμονόμησης
RC	♦ Recupero ♦ Corrélation ♦ Wiederfindung ♦ Recuperacão	♦ Recovery ♦ Recuperado ♦ Ανάκτηση
CONJ	♦ Coniugato ♦ Conjugé ♦ Konjugat ♦ Conjugado	♦ Conjugate ♦ Conjugado ♦ Σύζευγμα
MP	♦ Micropiastra rivestita ♦ Microplaque sensibilisée ♦ Beschichtete Mikrotiterplatte ♦ Microplaca revestida	♦ Coated microtiter plate ♦ Microplaca sensibilizada ♦ Επικαλυμμένη μικροπλάκα
PINP	♦ Piastra ad aghi rivestita ♦ Pinplate sensibilisée ♦ Beschichtete Pinplatte ♦ Pinplate revestida	♦ Coated pinplate ♦ Pinplate sensibilizada ♦ Επικαλυμμένη πλάκα Pin
WASHB 50x	♦ Tampone di lavaggio ♦ Tampon de Lavage ♦ Waschpuffer ♦ Solucão de lavagem	♦ Wash buffer ♦ Solución de lavado ♦ Ρυθμιστικό διάλυμα πλύσης
SUB	♦ Tampone substrato ♦ Substrat ♦ Substratpuffer ♦ Substrato	♦ Substrate buffer ♦ Tampón sustrato ♦ Ρυθμιστικό διάλυμα υποστρώματος
STOP	♦ Reagente bloccante ♦ Solution d'Arrêt ♦ Stopreagenz ♦ Solucão de paragem	♦ Stop solution ♦ Solución de parada ♦ Αντιδραστήριο διακοπής αντίδρασης
SB 5x	♦ Tampone campione ♦ Tampon Echantillons ♦ Probenpuffer ♦ Diluente de amostra	♦ Sample buffer ♦ Tampón Muestras ♦ Ρυθμιστικό διάλυμα δειγμάτων

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