***AESKULISA* tTg-G**

Protocol 30-30-30 REF30-7504US

Instruction manual

Contents

REF 30-7504US : Version 008: 2007-02-07

1. Intended Use

The ***AESKULISA*** **tTg-G** is a solid phase enzyme immunoassay for the semiquantitative and qualitative detection of IgG antibodies against tissue transglutaminase (tTG) in human serum.

The assay is an aid in the diagnosis of celiac disease (gluten-sensitive enteropathy) and should be used in conjuction with other serological tests and clinical findings.

2. Clinical Application and Principle of the Assay

Gluten-sensitive enteropathy or celiac disease is characterized by atrophy of the small intestinal villi leading to a so-called flat mucosa. It is caused by a pathological intolerance to gliadin, the alcohol-soluble fraction of gluten in wheat, rye and barley. The disease is HLA-associated (>95% of patients have DQ2 enREFd by DQA1*0501 and DQB1*0201) and manifests at any age with a peak onset in early childhood, even in neonatals.[6] The incidence rates range from 1 in 4000 to 1 in 300 in european countries.
Diagnosis of celiac disease is made by small intestinal biopsy (demonstrating flat mucosa) supported by serological markers. Antibodies against gliadin and anti-endomysium antibodies (EMA) are of major significance. They are detected so far by indirect immunofluorescence, which is restricted to subclass IgA only. The identification of tissue transglutaminase (tTg) as the major target antigen of EMA provided the opportunity of a more easy and reliable diagnosis of celiac disease.[1] tTG is an enzyme that upon wounding is released from cells where it is thought to aid in tissue repair.[2,3]
Anti-tTg antibodies show higher sensitivity and specificity than anti-Gliadin antibodies.[1] Furthermore they correlate tightly with the activity of the disease and thus are especially useful for diet monitoring. The cross-link of tTg with gliadin-specific peptides results in *neo-epitopes* of tTg. These *neo-epitopes* are structurally closer to the physiological antigens.[4,11] The AESKULISA tTg-G is coated with tTg crosslinked with gliadin-specific peptides.
The determination of IgG antibodies to tTg is the only available specific serology for those 2% to 5% of patients with IgA deficiency.[6] A high number of subclinical cases have been detected by screening for anti-tTg, fostering the theory that the majority of celiac disease cases is undetected and untreated (Iceberg model).[5]

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 next 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 next 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 anti-gen-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%

50x Wash Buffer — 1 vial, 20 ml - 50x concentrated (capped white: green solution)
Containing: Tris, NaCl, Tween-20, sodium azide < 0.1%

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 IgG (capped blue: blue solution)
Containing: PBS, BSA, Anti-human immunoglobulins conjugated to horseradish peroxidase

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
Coated with recombinant human tissue-Transglutaminase and Gliadin-specific peptides

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, 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.
The performance of the assay has not been established in the pediatric population.Though seven gliadin-positive (tTg-negative) sera have shown no crossreactivity with this assay, other gliadin antibody positive sera might show crossreactivity. Sera from patients with infectious diseases may also show crossreactivities.

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 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), in the dark.
- 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. Semi-Quantitative and Qualitative Interpretation

For **semi-quantitative 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 duplicate for each run.

Calibrators IgG	*OD 450/620 nm*	*CV % (Variation)*
0 U/ml	0.073	3.1
3 U/ml	0.179	2.3
10 U/ml	0.342	1.2
30 U/ml	0.662	0.1
100 U/ml	1.310	0.9
300 U/ml	2.263	0.3

Example of calculation

Patient	Duplicate (OD)	Mean (OD)	Result (U/ml)
P 01	0.808/0.831	0.820	39.6
P 02	1.081/1.071	1.076	66.1

For lot specific data, see enclosed quality control certificate. Medical laboratories might perform an in-house 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 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

The analytical sensitivity of this kit has been found at 1.0 U/ml.

10.2 Specificity and Sensitivity

The microplates are coated with **recombinant human tissue-transglutaminase and gliadin-specific peptides.** No crossreactivities to other autoantigens have been found. To test crossreactivity with gliadin, 7 sera positive for gliadin were tested and did not react with this assay, though this can be different for other gliadin positive sera.

For determination of sensitivity and specificity sera of 185 patients suffering from Celiac disease (n=122) and related diseases (data in bottom table) were assessed on the AESKULISA and a predicate device. The results as a comparison to the predicate device and disease information are shown in the tables below (The data has been aquired with the AESKULISA tTg G (REF7504US)).

		AESKULISA tTg-G		
		positive	negative	
diagnose	positive	80	4	84
	negative	5	96	101
		85	100	185

sensitivity: 95.2%
specificity: 95.1%
agreement: 95.1%

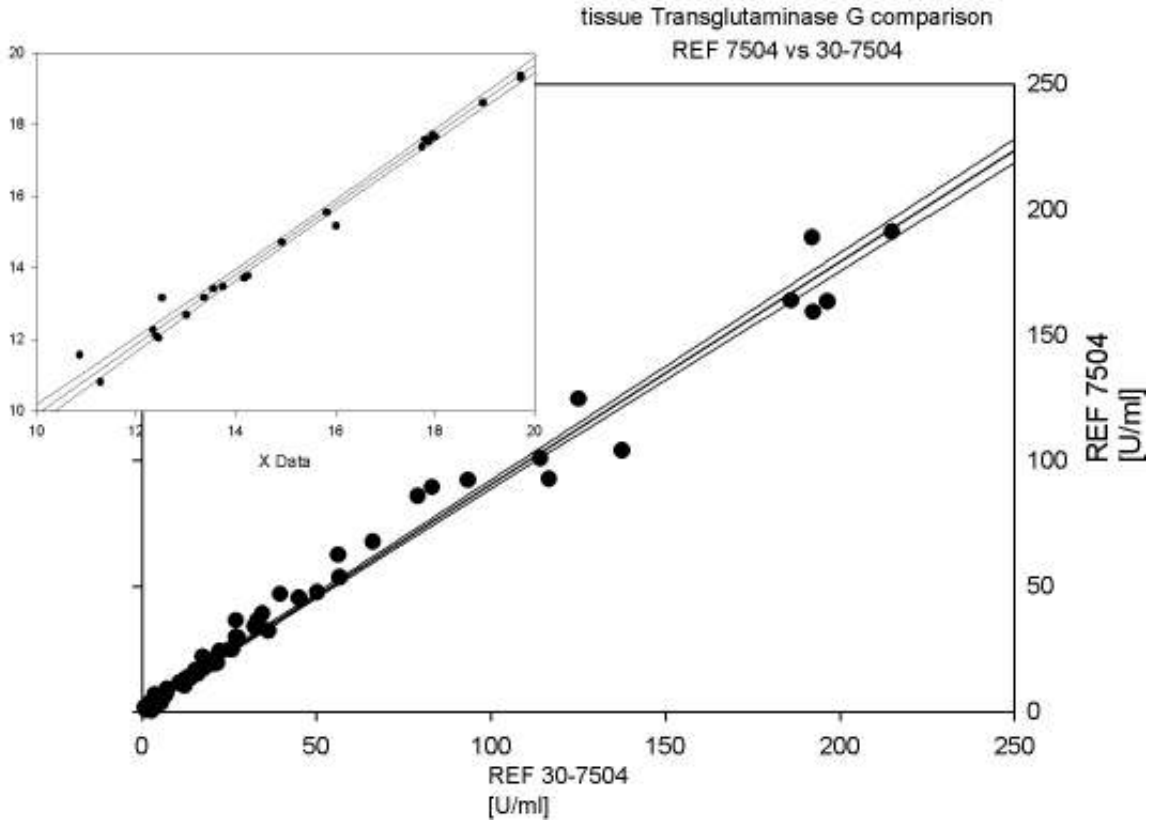
		positive	negative	
predicate device	positive	18	4	22
	negative	67	96	163
		85	100	185

rel. sensitivity: 81.8%
rel. specificity: 58.9%
rel. agreement: 61.6%

Disease	# Tested pred / AESKU	# positive pred. dev. (%)	# positive AESKU (%)
Celiac Disease	**64 / 64**	**9 (14.0)**	**59 (96.5)**
Celiac Disease (IgA deficient)	**20 / 20**	**9 (45.0)**	**18 (90.0)**
Celiac Disease (gluten free diet)	**38 / 38**	**0 (0.0)**	**0 (0.0)**
Disease control (total)	**70 / 217**	**4 (5.7)**	**11 (5.1)**
Crohns Disease	**51 / 51**	**2 (3.9)**	**3 (5.9)**
Crohns Disease	**0 / 58**	**n / d**	**0 (0.0)**
Ulcerative Colitis	**4 / 4**	**1 (25.0)**	**1 (25.0)**
Ulcerative Colitis	**0 / 2**	**n / d**	**1 (50.0)**
Helminthiasis	**2 / 2**	**1 (50.0)**	**2 (100)**
Lactose Intolerance	**2 / 2**	**0 (0.0)**	**2 (100)**
Gliadin positive sera	**0 / 7**	**n / d**	**0 (0.0)**
Healthy donors	**4 / 4**	**0 (0.0)**	**0 (0.0)**
Healthy donors	**0 / 80**	**n / d**	**2 (2.5)**

The data has been aquired with the AESKULISA tTg G (REF7504). The comparability of these data was assessed with 75 sera tested on both, REF7504US (30-15-15 minute protocol) and REF 30-7504US (30-30-30 protocol). A linear regression analysis of the two products showed that the two products are equivalent. (Range 10-20 U/ml is the panel upper left)

Y = b[0] + b[1]X	**value**	**range (CI95%)**
b[0]	**0.71**	**-0.08 / 1.50**
b[1]	**0.93**	**0.915 /0.945**
r^2	**0.99**	



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. Data has been measured with the 30 - 15 - 15 protocol (REF 7504US)

Sample No.	Dilution Factor	measured concentration (U/ml)	expected concentration (U/ml)	Recovery (%)
1	1 / 100	41.3	41.0	105.4
	1 / 200	22.2	20.5	108.3
	1 / 400	11.2	10.3	109.3
	1 / 800	5.5	5.1	107.1
2	1 / 100	86.6	87.0	99.5
	1 / 200	42.9	43.5	98.5
	1 / 400	23.9	21.8	109.7
	1 / 800	11.9	10.9	109.4

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. (n=18). (Range CV ≤ 10). Data has been measured with the 30 - 15 - 15 protocol (REF 7504US)

Intra-Assay			Inter-Assay		
Sample No.	Mean (U/ml)	CV (%)	Sample No.	Mean (U/ml)	CV (%)
1	19.2	3.1	1	16.7	4.3
2	100.8	3.5	2	97.4	7.6
3	152.8	4.6	3	194.4	9.1

10.5 Calibration

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

11. Literature

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Gliadin and tissue transglutaminase complexes in normal and coeliac duodenal mucosa.
Clin Exp Immunol. 134: 516-24.

ANNEX A: Pipetting scheme

We suggest pipetting calibrators, controls and samples as follows:

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

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

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

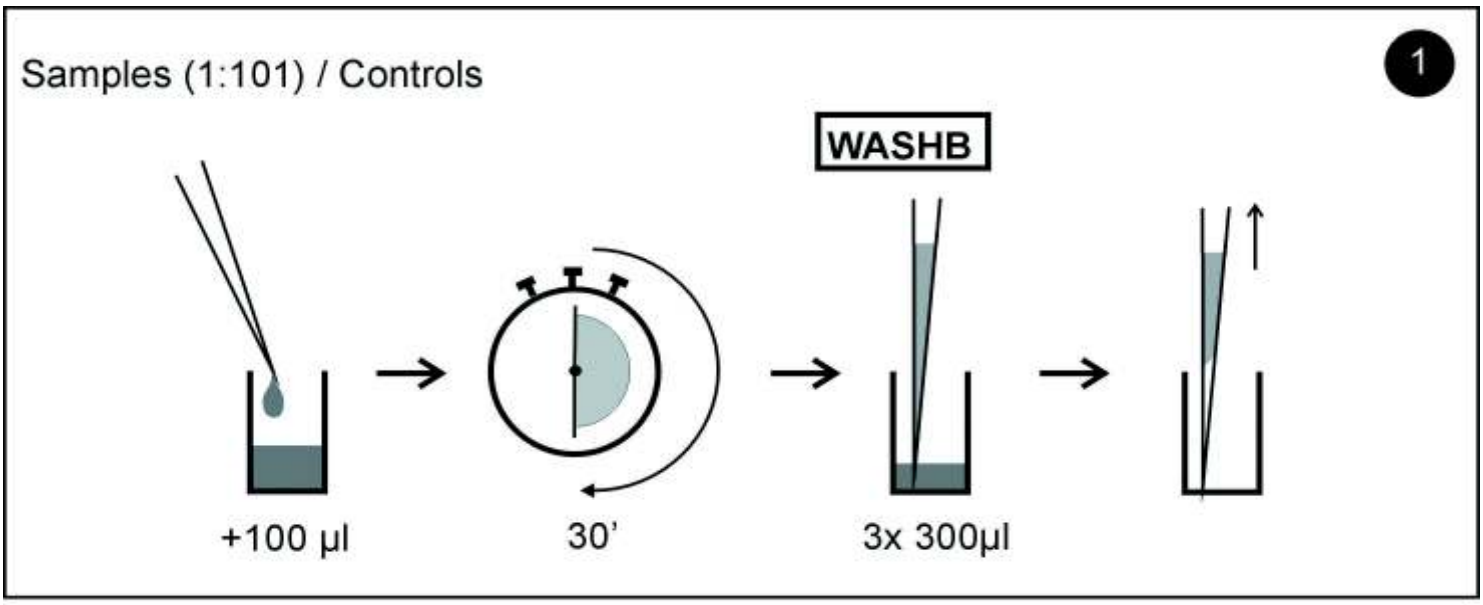
	1	2	3	4	5	
A	CalA	CalE	P1			
B	CalA	CalE	P1			
C	CalB	CalF	P2			
D	CalB	CalF	P2			
E	CalC	PC	P3			
F	CalC	PC	P3			
G	CalD	NC	...			
H	CalD	NC	...			

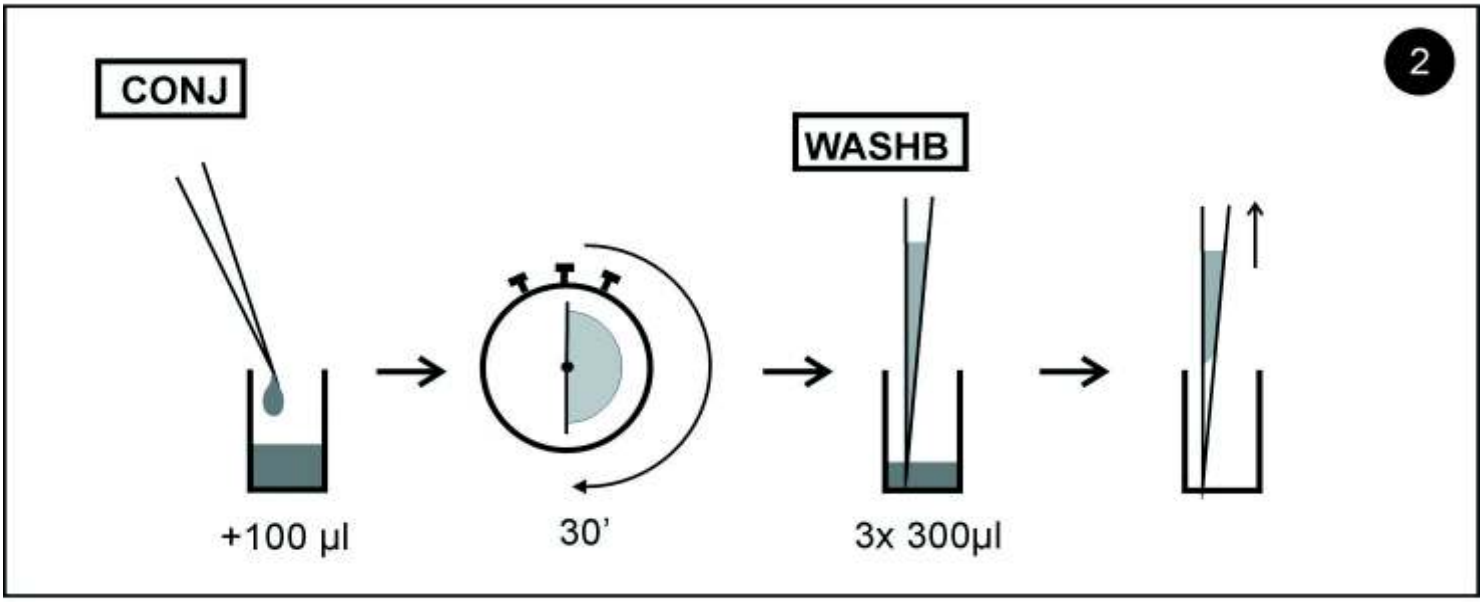
For **qualitative interpretation** use cut-off control

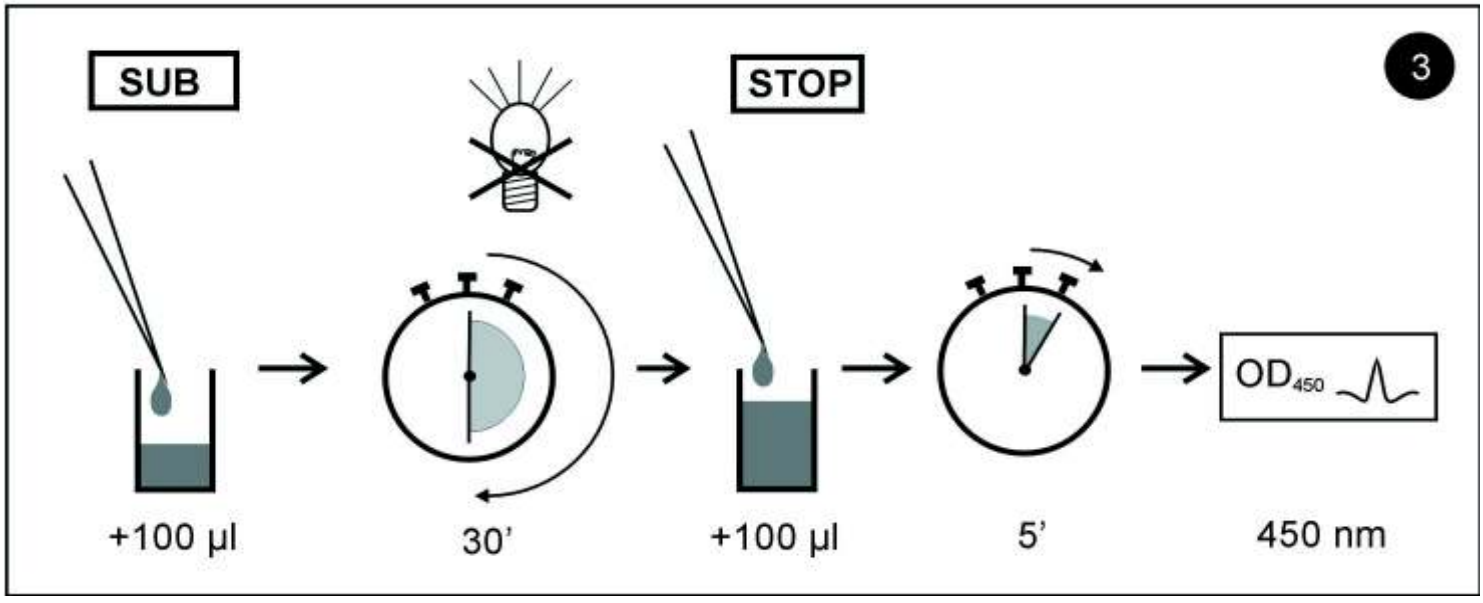
	1	2	3	4	5	
A	NC	P2				
B	NC	P2				
C	CC	P3				
D	CC	P3				
E	PC	...				
F	PC	...				
G	P1	...				
H	P1	...				

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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