AESKULISA **ANCA-Pro**

REF 3301

Instruction manual

Contents

1. Intended Use

AESKULISA ANCA Pro is a solid phase enzyme immunoassay employing highly purified native myeloperoxidase (MPO) and proteinase 3 (PR3) from human peripheral blood polymorphnuclear cells and native human Cathepsin G, Elastase, Lactoferrin, Lysozym and BPI (bacterial permeability-increasing protein) for the separate semi-quantitative and qualitative detection of antibodies against these antigens in human serum.
The assay is a tool in the diagnosis of autoimmune systemic vasculitis.

2. Clinical Application and Principle of the Assay

The acronym ANCA (Anti-neutrophil cytoplasmic autoantibodies) describes a group of antibodies directed against different components of neutrophilic granulocytes and monocytes. For the detection of ANCAs indirect immunofluorescence test on ethanol-fixed neutrophils has been the established method so far. It became apparent that some ANCAs create a cytoplasmic fluorescence pattern (thus called cANCA) while others create a perinuclear pattern (the pANCA). As both patterns may cover multiple antigens, immunofluorescence does not suffice for a satisfying differential diagnosis of vasculitis; thus each IFT should be verified with specific ELISA tests.

Myeloperoxidase (MPO) has been identified as the major pANCA antigen, but other cellular components like Lactoferrin, Cathepsin G, Lysozyme and Elastase cause perinuclear staining, too and therefore are included into the group of pANCAs. Proteinase 3 is the major target antigen of cANCA.

Detection of ANCAs is a useful laboratory diagnostic test for certain small vessel vasculitides and some non-vasculitic clinical syndromes, such as inflammatory bowel disease (IBD). Antibodies against MPO correlate with idiopathic or vasculitis associated necrotizing crescentic glomerulonephritis. They are found frequently in 70% of patients with microscopic polyangiitis as well as 5-50% of patients with Churg-Strauss syndrome. Antibodies against Lactoferrin and Cathepsin G were identified in a subgroup of patients with inflammatory bowel disease. However, these ANCA specificities do not appear to correlate with disease activity. Autoantibodies against Elastase are generally associated with inflammatory rheumatic diseases like SLE, Sjögren`s syndrome and Felty syndrome. Antibodies against BPI are detected in chronically infectious intestinal diseases such as Morbus Crohn or ulcerative colitis. In contrast to anti-MPO anti-BPI antibodies do not seem to have any association with vasculitis. Antibodies to Lysozyme occur in higher frequency in rheumatoid vasculitis and inflammatory bowel disease like ulcerative colitis.

Autoantibodies to PR3 are a specific serological marker for Wegener`s granulomatosis (WG).

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 20, sodium azide < 0.1% (preservative)

Ready to use:

Calibrators A-D 4 vials, each 1.5 ml 0, 10, 30, 100 U/ml
(color increasing with concentration: 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)

Conjugate 1 vial,15 ml IgG (capped blue: blue solution)
Containing: 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
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 µl) or adjustable multipipette (100-1000ml). 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 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 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.
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.

8. Qualitative and Semiquantitative 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.**

Example of interpretation
We recommend pipetting cut-off calibrator in parallel for each run.

Calibrators/IgG	OD 450/620 nm
0 U/ml	0.035 OD
10 /Uml	0.329 OD
30 U/ml	0.721 OD
100 U/ml	1.578 OD
Cut-off calibrator	
15 U/ml	0.45 OD

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

Example of calculation

Patient	Replicate (OD)	Mean (OD)	Result qualitative	Result (U/ml) semiquantitative
P 01	0.188/0.186	0.187	negative	5.0
P 02	1.334/1.335	1.335	positive	71.4

Do not use this example for interpreting patients results!

We recommend to retest samples, that are borderline. 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 EU regulations.

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

Qualitative Calculation
Calculation of the *AESKULISA* ANCAPro test can be carried out by direct comparison of the optical density (OD) of each patient sample with the optical density 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: $OD_{patient} < 0.8 \times OD_{cut\text{-}off}$

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

Positive $OD_{patient} > 1.2 \times OD_{cut\text{-}off}$

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 20-32°C/68-89.6°F
Calibration range:	0-100 U/ml
Analytical sensitivity:	1.0 U/ml
Storage:	at 2-8°C/35-46°F use original vials, only
Number of determinations:	12x8 tests

10. Performance Data

10.1 Analytical sensitivity

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

10.2 Specificity

The microplate is coated with highly purified native myeloperoxidase (MPO) and proteinase 3 from human peripheral blood polymorphnuclear cells and native human Cathepsin G, Elastase, Lactoferrin, Lysozym and BPI (bacterial permeability-increasing protein). No crossreactivities to other autoantigens have been found. The data has been aquired with the *AESKULISA ANCA-Pro (REF7301)*.

Correlation:

The comparability of performance data was assessed with at least 30 sera tested on both, AESKULISA 7301 and AESKULISA 3301. A linear regression analysis of the two products showed that the two products are equivalent. Data can be received upon request.

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.

Sample No.	Dilution Factor	measured concentration (U/ml)	expected concentration (U/ml)	Recovery (%)
1	1 / 100	223.0	225.0	99.1
	1 / 200	110.5	112.5	98.2
	1 / 400	57.3	56.3	101.8
	1 / 800	27.3	28.1	97.2
2	1 / 100	163.6	165.0	99.2
	1 / 200	82.5	82.5	100.5
	1 / 400	42.9	41.3	103.9
	1 / 800	21.9	20.6	106.3

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.

Intra-Assay		
Sample No.	**Mean (U/ml)**	**CV (%)**
PR3	136.0	3.5
MPO	72.6	3.7
BPI	33.4	4.8
Elastase	24.9	5.7
Cathepsin-G	105.0	3.4
Lysozyme	36.9	4.1
Lactoferrin	86.4	4.1

Inter-Assay		
Sample No.	**Mean (U/ml)**	**CV (%)**
PR3	128.0	3.5
MPO	70.9	3.7
BPI	35.3	4.8
Elastase	22.8	5.7
Cathepsin-G	110.0	3.4
Lysozyme	32.4	4.7
Lactoferrin	84.6	5.8

10.5 Calibration

Due to the lack of international reference calibration *AESKULISA* ANCA-Pro is calibrated in arbitrary units (U/ml).

11. Literature

1. **Falk, RJ Jennette JC (1988).**
   Antineutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necotizing and crescentic glomerulonephritis.
   N Engl, J Med 318: 1651-1657.
2. **Lüdemann J, Utecht B, Gross WL (1990).**
   Antineutrophil cytoplasm antibodies in Wegener`s granulomatosis recognize an elastinophil enzyme.
   J Exp Med 171: 375-362.
3. **Seibold F, Weber P, Klein R, Berg PA, Wiedmann KH (1992).**
   Clinical significance of antibodies against neutrophils in patients with inflammatory bowel disease and primary sclerosing cholangitis.
   Gut 33: 657-662.
4. **Gross WL, Hauschild S, Mistry N (1993)** .
   The clinical relevance of ANCA in vasulitis 7-11 5th International ANCA Workshop, Cambridge.
   Clin Exp Immunol 93 (Suppl. 1).
5. **Peen E, Almer S, Bodemar G, Ryden BO, Sjolin C, Tejle K, Skogh T (1993).**
   Antilactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis and Crohn`s disease.
   Gut 34: 56-62.

ANNEX A: Pipetting scheme

We suggest pipetting calibrators, controls and samples as follows:

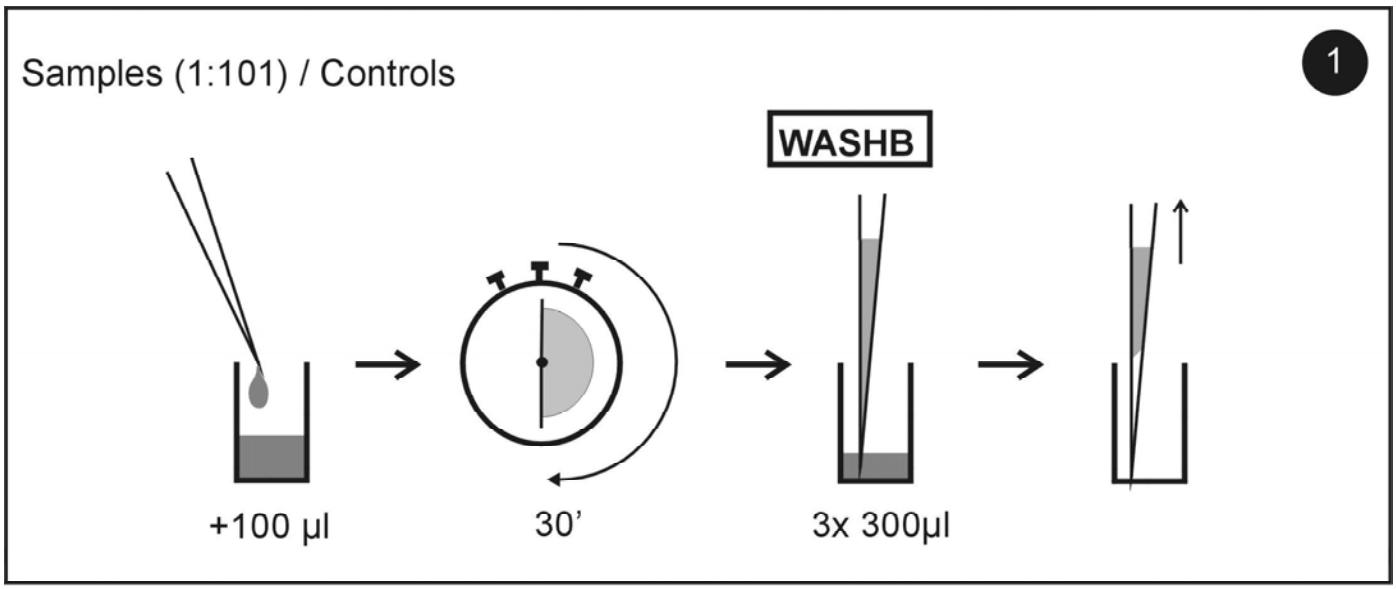
For **quantitative interpretation** use calibrators to establish a standard curve.

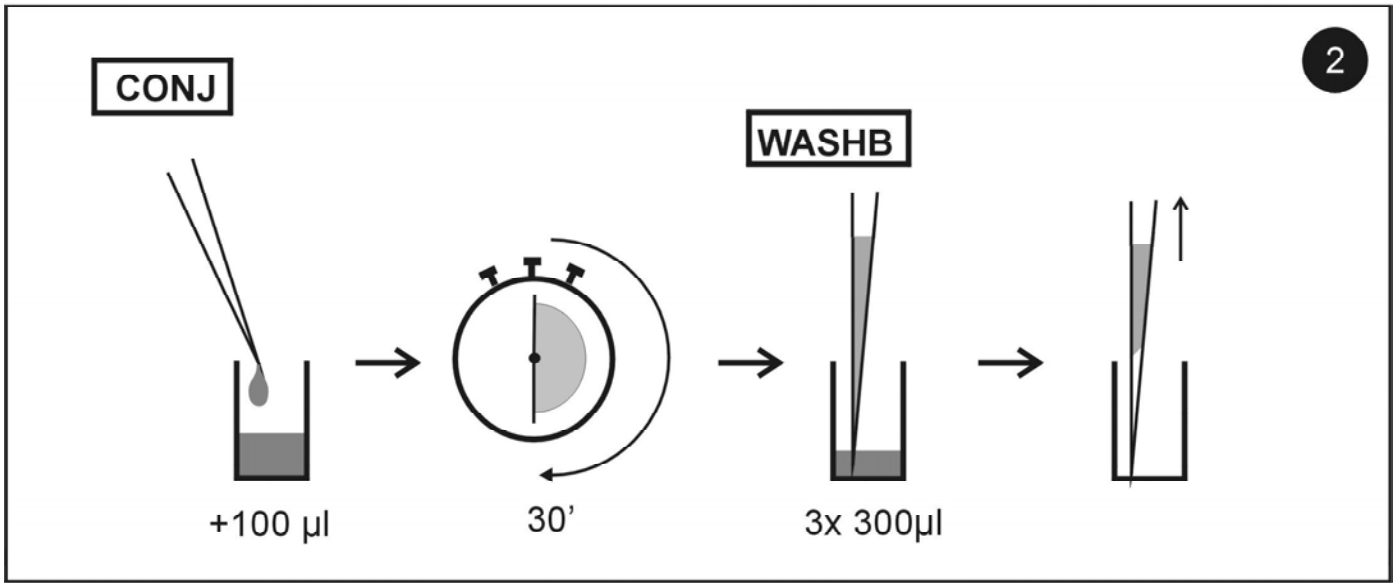
For **qualitative interpretation** use cut-off calibrator.

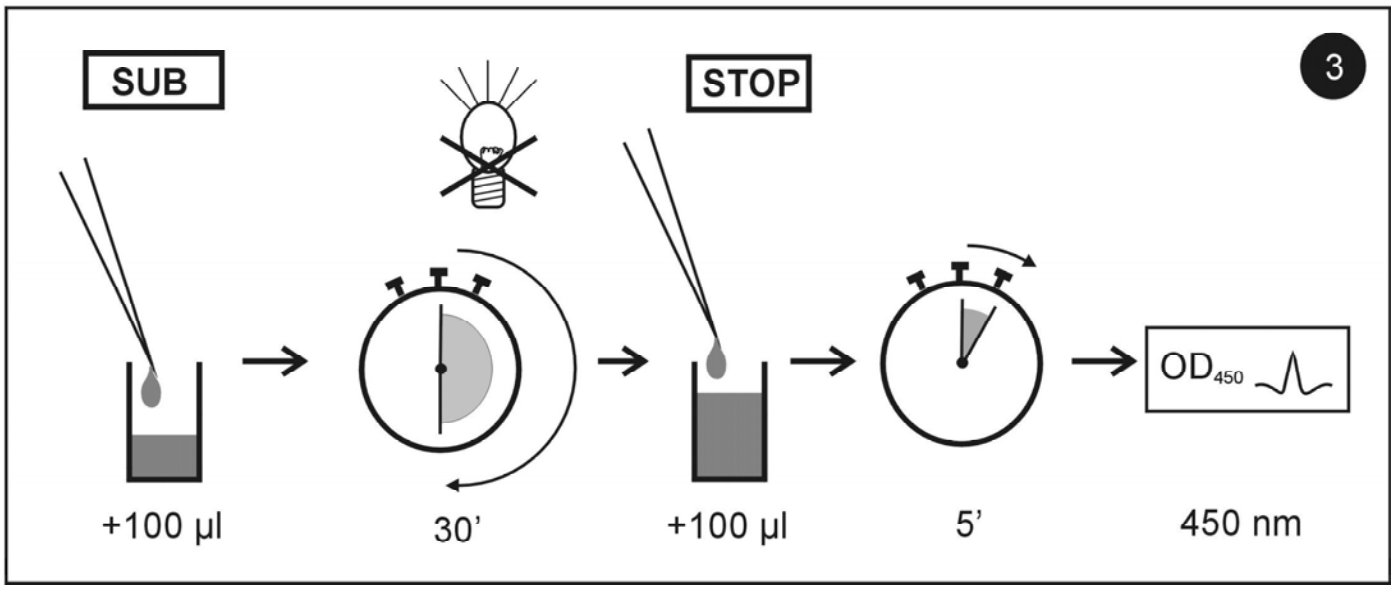
		for **quantitative interpretation** use calibrators to establish a standard curve						for **qualitative interpretation** use cut-off calibrator and CalA as negative control and CalD as positive control					
Antigen		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Cal. antigen	**A**	CalA	CalB	CalC	CalD			CalA	CC	CalD			
PR3	**B**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
MPO	**C**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
BPI	**D**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Elastase	**E**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Cathepsin G	**F**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Lysozym	**G**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Lactoferrin	**H**	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12

CalA: calibrator A, CalB: calibrator B, CalC: calibrator C, CalD: calibrator D
CC: Cut-off calibrator
P1: patient 1
P2: patient 2
P3: patient 3
Cal antigen: antigen coated for calibrators
PR3: Mroteinase 3
MPO: Myeloperoxidase
BPI: bacterial permeability-increasing protein

Annex B: Test Procedure







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
Calibration Antigen	**calibrator A (0 U /ml)**	**calibrator B (10 U /ml)**	**calibrator C (30 U /ml)**	**calibrator D (100 U /ml)**	**or alternative**	**cut off calibrator**						
PR3												
MPO												
BPI												
Elastase												
Cathepsin G												
Lysozym												
Lactoferrin												

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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