AESKULISA Protein C

REF 3901

Instruction manual

Contents

1. Intended Use

AESKULISA Protein C is a solid phase enzyme immunoassay for the quantitative determination of Protein C in citrated human plasma. The determination of Protein C aids in the risk estimation of thrombosis.

2. Clinical Application and Principle of the Assay

Protein C is a vitamin K-dependent inactive zymogen of a serine protease that is mainly synthesized by hepatocytes in the liver. It has a molecular weight of 62 kDa and is present at a concentration of 4 µg/ml in the plasma. Activated Protein C (aPC) is a key component of the Protein C anticoagulant system that is activated by the binding of thrombin to the endothelial transmembrane receptor thrombomodulin. The complex of thrombin and thrombomodulin activates Protein C and the activated Protein C in turn forms a complex with its cofactor Protein S that has a high affinity to phospholipid membranes. This is of physiological importance since aPC inactivates preferentially the membrane-bound coagulation factors Va and VIIIa. Additionally, activated Protein C possesses profibrinolytic activity by inhibiting plasmin activator inhibitor-1 (PAI-1). Protein C deficiency may be inherited or acquired and is associated with a variably increased risk of thrombosis. The prevalence of Protein C deficiency has been estimated to be up to one case per 300 in the general population. Nearly 50-80 % of individuals with inherited Protein C deficiency will experience a thrombotic event before the age of 30-45. Patients with a homozygous Protein C deficiency may suffer from neonatal purpura fulminans or massive venous thrombosis. Acquired Protein C deficiency is often associated with liver disease, surgery, oral anticoagulant therapy, antiphospholipid syndrome, etc. Protein C deficiency is classified in two states. Type I deficiency is a reduction in the level of Protein C. Type II deficiency is characterized by a reduced Protein C activity, with normal antigen level. To determine the type of defect, the laboratory diagnosis of Protein C may require both antigen levels and functional determination.

Principle of the test

The *AESKULISA* Protein C is a sandwich ELISA using microplates coated with a capture antibody specific for human Protein C. 1:51 diluted patient plasma is incubated in the wells allowing Protein C present in the plasma to bind to the antibody. The unbound fraction is removed by washing. Afterwards anti-human Protein C detection antibody conjugated to horseradish peroxidase (conjugate) is incubated and reacts with the antigen-antibody complex on the microwell surface. Following incubation, unbound conjugate is washed off. 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 measured in optical density units with a spectrophotometer at 450 nm. Using a curve prepared from the Reference Plasma provided wih the kit, the Protein C antigen relative percent concentration in patient plasma can be determined.

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)

Reference Plasma 3 vials, 0.4 ml lyophilized Reference Plasma
Containing: Human plasma

Control „N“ 3 vials, 0.2 ml lyophilized Normal Plasma
Containing: Human plasma

Control „D“ 3 vials, 0.2 ml lyophilized Deficient Plasma
Containing: Human plasma

Ready to use:

Conjugate 1 vial,15 ml IgG (capped blue: blue solution)
Containing: anti-human Protein C antibody 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 except for the Reference Plasma and the Controls are stable for 1 month at 4°C/39°F, at least. After reconstitution the Reference Plasma and the Controls are stable for 8 hours when stored at 2-8°C/35-46°F. ***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 ! 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.
The Reference Plasma and the Controls included in this kit have 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 Reference Plasma, Controls 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/68-78.8°F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Incubation: We recommend test performance at 23°C/73.4°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 plasma samples freshly collected with 3.2% or 3.8% sodium citrate as an anticoagulant. Blood withdrawal must follow national requirements. Do not use icteric, lipemic, hemolysed or bacterially contaminated samples. Blood samples should be collected in clean, dry and empty tubes. After centrifugation, the plasma samples should be used immediately, otherwise stored tightly closed at 2-8°C/35-46°F up to eight hours, 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).

Reference Plasma:
Reconstitute Reference Plasma by adding 0.4 ml distilled water and shake gently. Allow the reconstituted plasma to stand for 10 minutes at room temperature before use. The Reference Plasma is stable for 8 hours when stored at 2-8°C/35-46°F.

Controls:
Reconstitute Control N and Control D by adding 0.2 ml distilled water and shake gently. Allow the reconstituted Controls to stand for 10 minutes at room temperature before use.
The Controls are stable for 8 hours when stored at 2-8°C/35-46°F.

Predilution of the Reference Plasma:
Prepare a 1:2 dilution of reconstituted reference plasma in prediluted sample buffer (1x) and mix well, e.g. 100 µl sample buffer + 100 µl palsma.

Preparation of the reference curve:
The dilution set is prepared by using the prediluted Reference Plasma.

Volume Reference Plasma	Volume Sample Buffer	Reference Level
60 µl	1000 µl	150 %
40 µl	1000 µl	100 %
30 µl	1000 µl	75 %
20 µl	1000 µl	50 %
10 µl	1000 µl	25 %
10 µl	2000 µl	12.5 %

Dilution of the Samples and Controls:
Add 20 µl plasma to 1000 µl sample buffer (1x) and 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, Controls and working dilutions of the Reference Plasma in duplicate.

- Pipette 100 µl of each patient‘s diluted plasma into the designated microwells.
- Pipette 100 µl of each working dilution of the Reference Plasma and the diluted Controls into the designated wells.
- Incubate for 30 minutes at 20-26°C/68-78.8°F.
- Wash 3x with 300 µl washing buffer (diluted 1:50).
- Pipette 100 µl conjugate into each well.
- Incubate for 30 minutes at 20-26°C/68-78.8°F.
- Wash 3x with 300 µl washing buffer (diluted 1:50).
- Pipette 100 µl TMB substrate into each well.
- Incubate for 30 minutes at 20-26°C/68-78.8°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. Quantitative Interpretation

For **quantitative interpretation** establish the reference curve by plotting the **optical density (O.D.) of each dilution of the Reference Plasma (y-axis) against the corresponding value of the Reference Level in % (x-axis)**. For best results we recommend log/lin coordinates and 4-Parameter Fit. From the O.D. of each sample, read the corresponding patient relative value expressed in **%**. Multiply the patient relative value obtained from the reference curve by the assigned factor referred in the quality control leaflet to calculate the Protein C antigen level in % of normal.

Example of a reference curve

We recommend pipetting each dilution of the Reference Plasma in parallel for each run.

Reference Level	*OD 450/620 nm*	*Results (%)*	*CV % (Variation)*
12.5 %	0.569	11.95	1.05
25 %	0.874	26.68	0.94
50 %	1.163	48.06	1.04
75 %	1.434	77.70	0.97
100 %	1.583	99.61	1.01
150 %	1.826	147.73	1.02

Example of calculation

Patient	**Replicate (OD)**	**Mean (OD)**	**Patient relative value (%)**	**Factor**	**Patient Protein C (%)**
P 01	0.933/0.927	0.930	31.8	0.96	30.5
P 02	1.860/1.866	1.863	112.3	0.96	107.8

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 plasma, as foreseen by EU regulations.

Do not use this example for interpreting patients results!

Expected values

The values for Protein C are given in relative percent (%) as compared to pooled normal plasma. The Protein C concentration in normal human plasma ranges usually between 70 % and 140 %.
Samples with values above the range of the reference curve may be assayed again at higher dilutions for accurate 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.

9. Technical Data

Sample material:	plasma
Sample volume:	20 µl plasma diluted 1:51 with 1x sample buffer
Total incubation time:	90 minutes at 20-26°C/68-78.8°F
Calibration range:	12.5-150 %
Analytical sensitivity:	6.0 %
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 Protein C* gave an analytical sensivity of 6.0 %.

10.2 Specificity

The microplate is coated with **an antibody specific for human Protein C.**

10.3 Linearity

Chosen plasma have been tested with this kit and found to dilute linearly.

Sample No.	Dilution Factor	measured concentration (%)	expected concentration (%)	Recovery (%)
1	1 / 50	115.30	120	96.1
	1 / 100	60.88	60	101.5
	1 / 200	31.71	30	105.7
	1 / 400	14.41	15	96.1
2	1 / 50	41.47	40	103.7
	1 / 100	19.86	20	99.3
	1 / 200	9.48	10	94.8
	1 / 400	4.85	5	97.0

10.4 Precision

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

Intra-Assay		
Sample No.	Mean (%)	CV (%)
1	115.0	5.3
2	93.0	1.7
3	27.0	2.1

Inter-Assay		
Sample No.	Mean (%)	CV (%)
1	116.2	2.4
2	43.3	7.4
3	8.1	3.7

10.5 Calibration

This quantitative assay is calibrated against the WHO second international standard for Protein C. The values are given in relative percent (%) as compared to pooled normal plasma.

11. Literature

1. **Dahlbäck B, Villoutreix BO (2005).**
   The anticoagulant protein C pathway.
   FEBS Letters 579: 3310-3316.

2. **Esmon CT (2003).**
   The Protein C Pathway.
   Chest 124: 26-32.

3. **Miletich JP (1990).**
   Laboratory diagnosis of Protein C deficiency.
   Seminars in Thrombosis and Hemostasis 16: 169-176.

4. **Griffin JH, Evatt B, Wideman C, Fernandez JA (1993).**
   Anticoagulant Protein C Pathway defective in majority of thrombophilic patients.
   Blood 82: 1989-1993.

5. **Preissner KT (1990).**
   Biological relevance of the Protein C system and laboratory diagnosis of Protein C and S deficiencies.
   Clinical Science 17: 351-364.

ANNEX A: Pipetting scheme

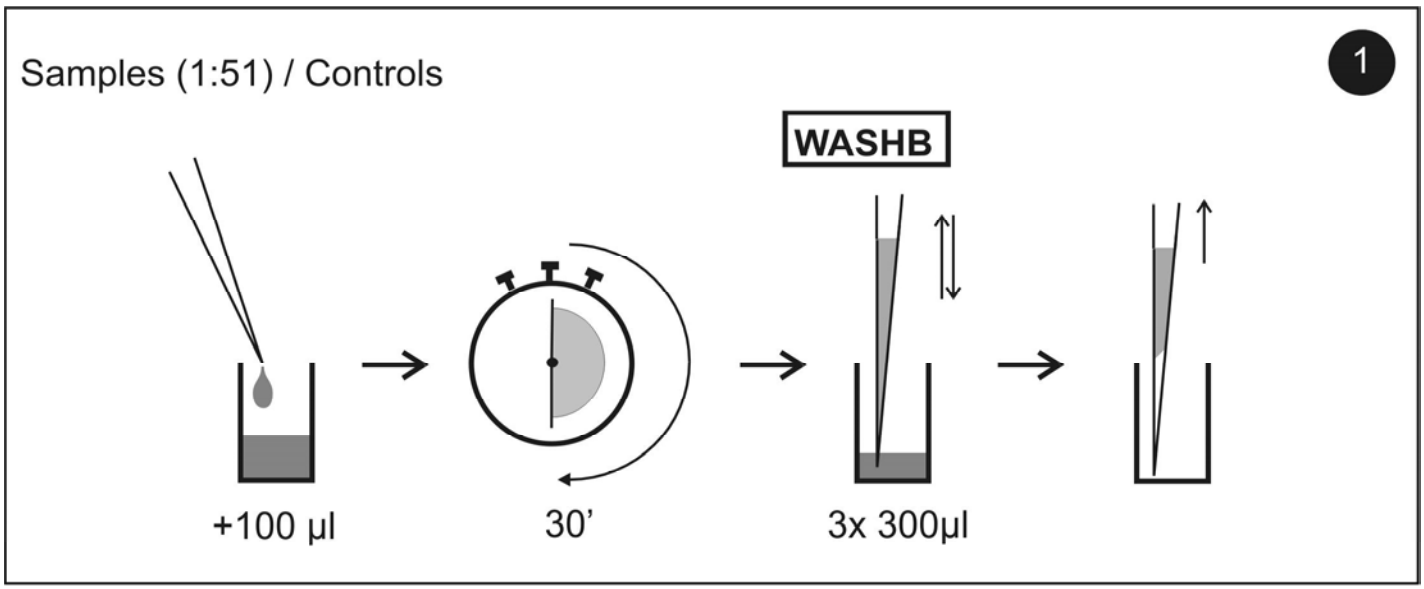
We suggest pipetting working dilutions of the Reference Plasma, controls and samples as follows:

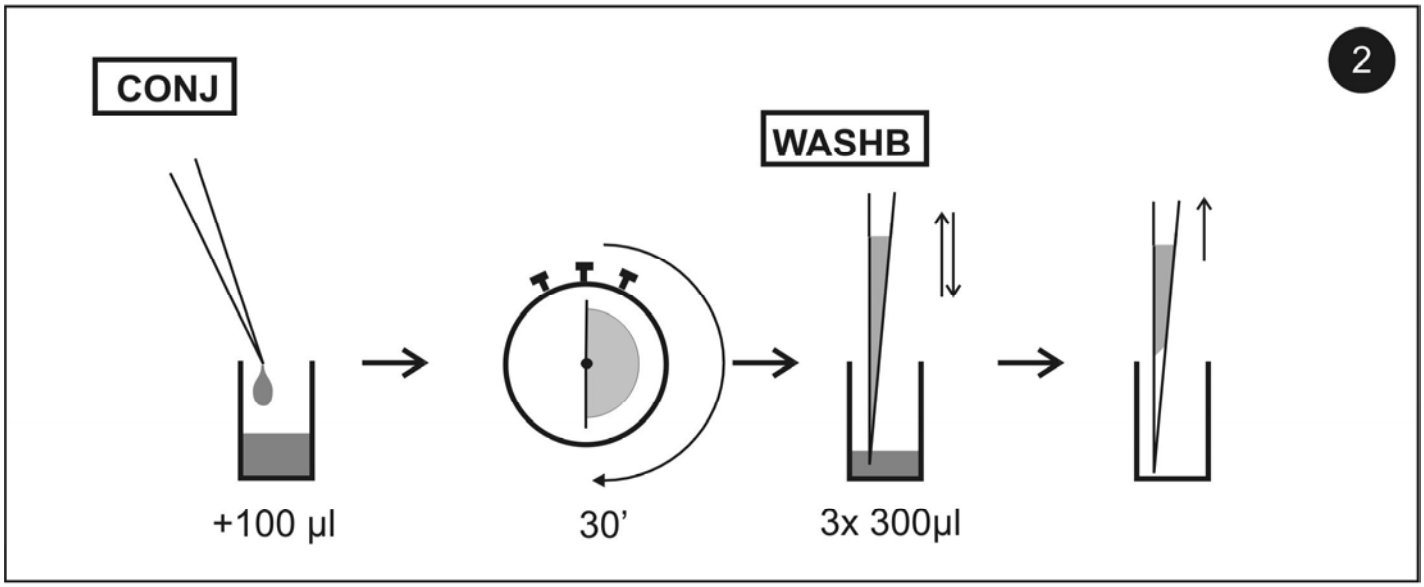
For **quantitative interpretation** use the working dilutions of the Reference Plasma to establish a standard curve.

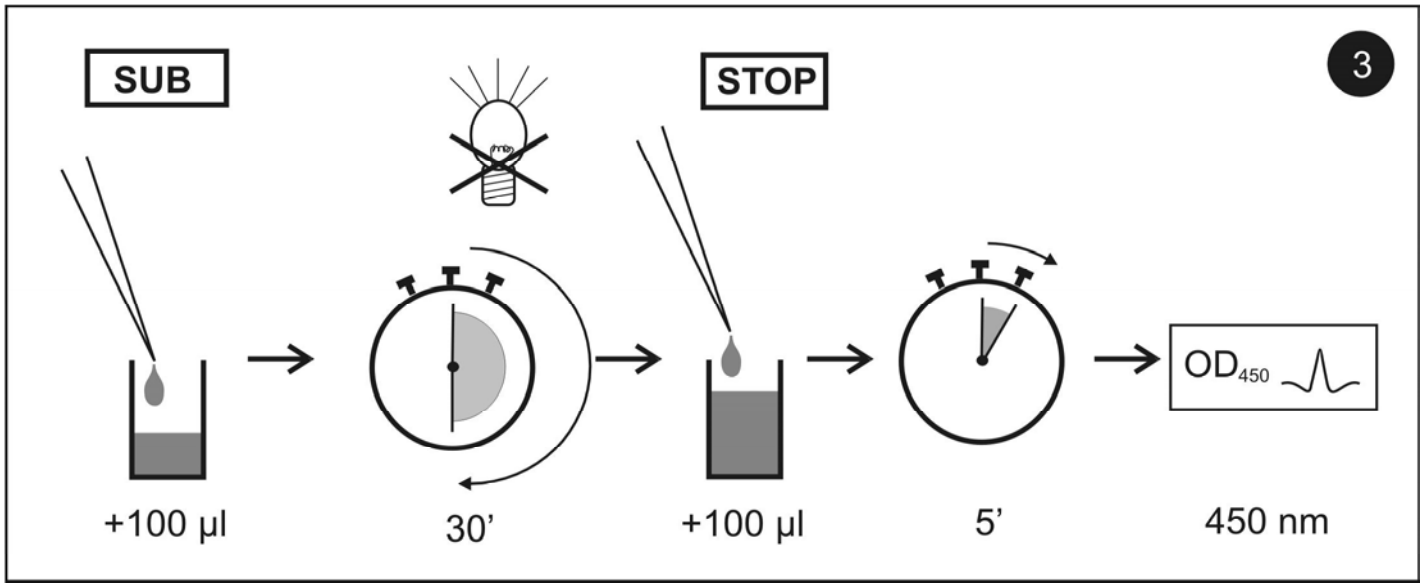
	for **quantitative interpretation** use the working dilutions of the Reference Plasma to establish a standard curve											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	150	25	P1									
B	150	25	P1									
C	100	12.5	P2									
D	100	12.5	P2									
E	75	CD	P3									
F	75	CD	P3									
G	50	CN	...									
H	50	CN	...									

150: Reference Level 150 %, 100: Reference Level 100 %, 75: Reference Level 75 %, 50: Reference Level 50 %, 25: Reference Level 25 %, 12.5: Reference Level 12.5 %
CD: control ‚deficient plasma'
CN: control ‚normal plasma'
P1: patient 1
P2: patient 2
P3: patient 3

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
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
+8°C +2°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
CON N	♦ Controllo con plasma normale ♦ Contrôle de plasma normal ♦ Kontrolle Normalplasma ♦ Controlo Plasma normal	♦ Control Normal Plasma ♦ Control Plasma normal ♦ Μάρτυρας Φυσιολογικού πλάσματος
CON D	♦ Controllo con plasma carente ♦ Contrôle de plasma déficient ♦ Kontrolle Mangelplasma ♦ Controlo Plasma deficiente	♦ Control Deficient Plasma ♦ Control Plasma deficiente ♦ Μάρτυρας Παθολογικού πλάσματος
CONJ	♦ Coniugato ♦ Conjugé ♦ Konjugat ♦ Conjugado	♦ Conjugate ♦ Conjugado ♦ Σύζευγμα
MP	♦ Micropiastra rivestita ♦ Microplaque sensibilisée ♦ Beschichtete Mikrotiterplatte ♦ Microplaca revestida	♦ Coated microtiter plate ♦ Microplaca sensibilizada ♦ Επικαλυμμένη μικροπλάκα
PEG	♦ Polietilenglicole ♦ Polyéthylèneglycol ♦ Polyethylenglykol ♦ Polietilenglicol	♦ Polyethylenglycol ♦ Polietilenglicol ♦ Πολυεθυλενγλικόλη
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
Ref. Plasma	♦ Plasma di riferimento ♦ Plasma de référence ♦ Referenzplasma ♦ Plasma de referência	♦ Reference Plasma ♦ Plasma de referencia ♦ Πλάσμα αναφοράς