AESKULISA Prothrombin-A

Instruction manual

Contents

1. Intended Use	1
2. Clinical Applications and Principle of the Assay	1
3. Kit Contents	2
4. Storage and Shelf Life	2
5. Precautions of Use	3
6. Sample Collection, Handling and Storage	3
7. Assay Procedure	4
8. Quantitative and Qualitative Interpretation	5
9. Technical Data	6
10. Performance Data	6-7
11. Literature	7
A : Pipetting scheme	8
B : Test Procedure	9

002 : 2007-08-28 REF 3210 Prothrombin-A

1. Intended Use

AESKULISA Prothrombin-A is a solid phase enzyme immunoassay employing native human prothrombin (Factor II) for the quantitative and qualitative detection of IgA antibodies against prothrombin in human serum.

The assay is a tool in the diagnosis of the antiphospholipid syndrome.

2. Clinical Application and Principle of the Assay

Human Factor II (prothrombin) is a plasme zymogen with a molecular weight of 72 kDa. It assembles with the activated forms of Factor V, Factor X and phospholipid to form a catalytic unit known as the prothrombinase complex. In the presence of calcium ions, the complex cleaves the membrane-associated prothrombin into thrombin, which is then released into the soluble phase.

Antibodies against prothrombin belong to the group of antiphospholipid antibodies specific for negatively charged phospholipids, components of biological membranes, and prothrombin. The presence of anti-phospholipid antibodies has been associated with the clinical features of the so-called antiphospholipid syndrome (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 infarct). The exact mechanism by which pathogenic anti-phospholipid antibodies induce thrombosis is not yet revealed fully.

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 anti-phospholipid syndrome (APS).

Antibodies targeting prothrombin alone have recently been described being highly associated with fetal loss in APS patients. Prothrombin antibodies are the first marker for this serious complication as all other known antiphospholipid antibodies failed to correlate with fetal loss.

Additionally, antibodies targeting a complex of prothrombin and phosphatidylserine have been associated with the second major group of clinical manifestations of the APS, venous and arterial thrombosis. However, they failed to show any correlation with fetal loss.

Therefore, both types of antibodies serve as an important diagnostic tool for differential diagnosis of the heterogenous manifestations of the APS.

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.

Page 1 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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)

Conjugate 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.*

Page 2 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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.

Page 3 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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.

Page 4 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

8. Quantitative and Qualitative Interpretation

For **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	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	0.014	2.9
3 U/ml	0.138	1.0
10 U/ml	0.283	1.5
30 U/ml	0.620	2.3
100 U/ml	1.314	0.9
300 U/ml	2.070	1.2

Example of calculation

Patient	Replicate (OD)	Mean (OD)	Result (U/ml)
P 01	0.547/0.555	0.551	24.3
P 02	1.638/1.641	1.640	167.4

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

Page 5 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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 Prothrombin-A* gave an analytical sensivity of 1.0 U/ml.

10.2 Specificity and sensitivity

The microplate is coated with **native human prothrombin**. No crossreactivities to other autoantigens have been found. Up to 50 % of pregnant women with APS suffer from fetal loss. *AESKULISA* Prothrombin identifies 84 % of those.

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	239.0	244.0	98.0
	1 / 200	120.0	122.0	98.4
	1 / 400	59.0	61.0	96.7
	1 / 800	29.4	30.5	96.4
2	1 / 100	172.0	178.0	96.6
	1 / 200	85.0	89.0	95.6
	1 / 400	42.0	44.5	94.3
	1 / 800	20.3	22.3	91.0

Page 6 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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 Mean CV									
No.	(U/ml)	(%)							
1	256.0	1.2							
2	138.0	3.4							
3	64.0	3.3							

Inter-Assay								
Sample Mean CV								
No.	(U/ml)	(%)						
1	239.0	1.4						
2	155.0	3.3						
3	58.0	3.2						

10.5 Calibration

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

11. Literature

1. Furie B and Furie BC (1988)

The molecular basis of blood coagulation.

Cell 53: 505-518.

2. Mann KG, Nesheim ME, Tracy PB, Hibbard LS, Bloom JS (1982).

Assembly of the prothrombinase complex.

Biophys J 37: 106-107.

3. Boey, ML, Colaco, CB, Gharavi, AE, et al. (1983):

Thrombosis in systemic lupus erythematosus: striking association with the presence of circulating lupus anticoagulant.

Br. Med. J. 287, 1021-1023.

4. T. Atsumi, M. Ieko, M.L. Bertolaccini, K. Ichikawa, A. Tsutsumi, E. Matsuura, T. Koike (2000).

Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of the lupus anticoagulant.

Arthritis & Rheumatism 43: 1982-1993.

5. P. von Landenberg, T. Matthias, J. Zaech, M. Schultz, M. Lorber, M. Blank, Y. Shoenfeld (2003).

Antiprothrombin antibodies are associated with pregnancy loss in patients with the antiphospholipid syndrome.

Am J Reprod Immunol 49: 51-56.

Page 7 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

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						alitativ ibrator	e inter	pretati	on use	cut-	
	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					

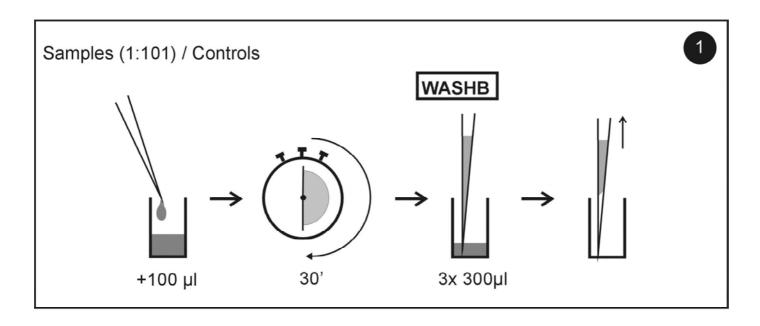
CalA: calibrator A, CalB: calibrator B, CalC: calibrator C, CalD: calibrator D, CalE: calibrator E,

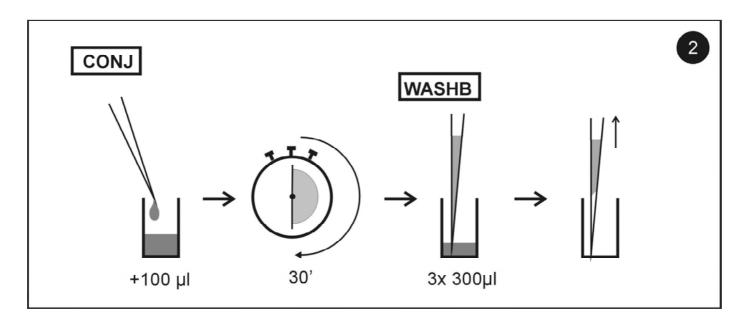
CalF: calibrator F PC: positive control NC: negative control CC: Cut-off calibrator

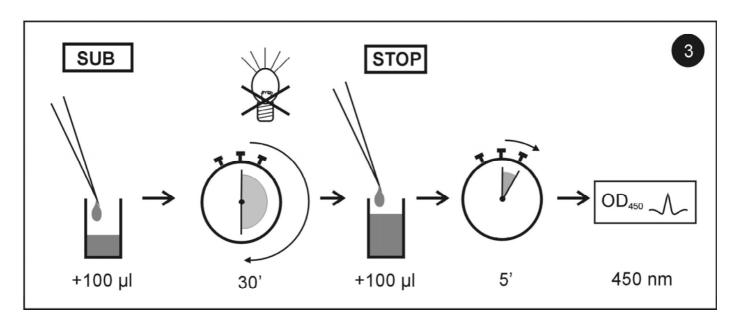
P1: patient 1 P2: patient 2 P3: patient 3

Page 8 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

Annex B: Test Procedure







Page 9 of 9 002 : 2007-08-28 REF 3210 Prothrombin-A

Incubation / Inkub. : 1min Date/ Datum:	°C 2. min	3min	4 5 6 7 8 9 10 11 12								
Incubation /			4								
	Ъ		3								
	emperature/Temperatur:		1 2								
ssay/Test:	emperature/	Vame:		A	В	C	D	Щ	Ħ	Ð	

AESKU.DIAGNOSTICS GmbH 55234 Wendelsheim - Mikroforum Ring 2, Germany Phone: + 49-6734-96270, Fax: + 49-6734-962727

	L . 5:	
13.45	Diagnosi in vitro Pour diagnostic in vitro	◆ For in vitro diagnostic use ◆ Para uso diagnostico in vitro
I IVD I	◆ Pour diagnostic in vitro◆ In Vitro Diagnostikum	 ◆ Para uso diagnóstico in vitro ♦ In Vitro Διαγνωστικό μέσο
	◆ Para uso Diagnóstico in vitro	τ τ.ασ Διαγνωστικό μέσο
	♦ Numero d'ordine	◆ Cataloge number
חרר	♦ Référence Catalogue	◆ Numéro de catálogo
REF	♦ Bestellnummer	 Αριθμός παραγγελίας
	Número de catálogo	
	◆ Descrizione lotto	♦ Lot
LOT	♦ Lot	♦ Lote
	♦ Chargen Bezeichnung	 Χαρακτηρισμός παρτίδας
	◆ Lote ◆ Conformità europea	◆ EC Declaration of Conformity
CE	◆ Déclaration CE de Conformité	Declaration of Conformity Declaración CE de Conformidad
7	Europäische Konformität	 Συρωπαϊκή συμφωνία
	 ◆ Déclaração CE de Conformidade 	
	♦ 96 determinazioni	♦ 96 tests
\96/	♦ 96 tests	♦ 96 pruebas
	♦ 96 Bestimmungen	96 προσδιορισμοί
	♦ 96 Testes	
	♦ Rispettare le istruzioni per l'uso	♦ See instructions for use
l lil	 ◆ Voir les instructions d'utilisation ◆ Gebrauchsanweisung beachten 	♦ Ver las instrucciones de uso
	◆ Ver as instrucões de uso	Λάβετε υπόψη τις οδηγίες χρήσης
	Ver as instrucces de uso Da utilizzarsi entro	♦ Use by
l J	♦ Utilise avant le	♦ Utilizar antes de
≟	 Verwendbar bis 	Χρήση μέχρι
	◆ Utilizar antes de	
∩ ~+8℃	♦ Conservare a 2-8°C	♦ Store at 2-8°C (35-46°F)
	♦ Conserver à 2-8°C	♦ Conservar a 2-8°C
+2.c~	♦ Lagerung bei 2-8°C	♦ Φυλάσσεται στους 2-8°C
	♦ Conservar entre 2-8°C	
_	♦ Prodotto da	♦ Manufactured by
	◆ Fabriqué par	◆ Fabricado por
	◆ Hergestellt von◆ Fabricado por	 Κατασκευάζεται από
	Calibratore cut-off	◆ Cut off Calibrator
CO CAI	Etalon Seuil	Calibrator Calibrator
JUU-CAL	Grenzwert Kalibrator	 ◆ Οριακός ορός Αντιδραστήριο βαθμονόμησης
	◆ Calibrador de cut-off	
	♦ Controllo positivo	◆ Positive Control
CON +	◆ Contrôle Positif	◆ Control Positivo
CONT	◆ Positiv Kontrolle	 Θετικός ορός ελέγχου
	◆ Controlo positivo	
	♦ Controllo negativo	♦ Negative Control
CON] -	◆ Contrôle Négatif	◆ Control Negativo
0014	Negativ KontrolleControlo negativo	 Αρνητικός ορός ελέγχου
	Collition regativo Calibratore	♦ Calibrator
041	♦ Etalon	◆ Calibrator
CAL	♦ Kalibrator	 Αντιδραστήριο βαθμονόμησης
	◆ Calibrador	
	♦ Recupero	♦ Recovery
RC	◆ Corrélation	♦ Recuperado
110	♦ Wiederfindung	♦ Ανάκτηση
	♦ Recuperacão	
	♦ Conjugato	◆ Conjugate
CONJ	◆ Conjugé ◆ Konjugat	 Conjugado Σύζει γιμα
	♦ Konjugat♦ Conjugado	Σύζευγμα
	Micropiastra rivestita	◆ Coated microtiter plate
NAD	Microplague sensibilisée	Microplaca sensibilizada
MP	Beschichtete Mikrotiterplatte	 ◆ Επικαλυμμένη μικροπλάκα
	 Microplaca revestida 	
	◆ Piastra ad aghi rivestita	◆ Coated pinplate
PINP	◆ Pinplate sensibilisée	◆ Pinplate sensibilizada
PINE	◆ Beschichtete Pinplatte	◆ Επικαλυμμένη πλάκα Pin
	♦ Pinplate revestida	
	Tampone di lavaggio Tampon de lavaggio	♦ Wash buffer
WASHB 50x	◆ Tampon de Lavage◆ Waschpuffer	 Solución de lavado ♣ Puθυιστικό διάλυμα πλύσης
1 1 10 1 15 00 K	◆ Wascripulier ◆ Solucão de lavagem	♦ Ρυθμιστικό διάλυμα πλύσης
	Tampone substrato	♦ Substrate buffer
21.5	Substrat	◆ Tampón sustrato
SUB	Substratpuffer	 ◆ Ρυθμιστικό διάλυμα υποστρώματος
	◆ Substrato	, , , , , , , , , , , , ,
	♦ Reagente bloccante	◆ Stop solution
CTOD	♦ Solution d'Arrêt	♦ Solución de parada
STOP	◆ Stopreagenz	 Αντιδραστήριο διακοπής αντίδρασης
	♦ Solução de paragem	
	◆ Tampone campione	♦ Sample buffer
SB 5x	◆ Tampon Echantillons	◆ Tampón Muestras
JD JX	◆ Probenpuffer	 ◆ Ρυθμιστικό διάλυμα δειγμάτων
	 Diluente de amostra 	