



**REF 10301**  
**ANCA-Pro**



# Instruction Manual

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	Product Ref.	10301
	Product Desc.	ANCA-Pro
	Manual Rev. No.	005 : 2014-01-15

## 1 Intended Use

**ANCA Pro** is a solid phase enzyme immunoassay employing highly purified native myeloperoxidase (MPO) and proteinase 3 (PR3) from human peripheral blood polymorphonuclear 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 intensity 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.

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### 3 Kit Contents

<b>TO BE RECONSTITUTED</b>				
Item	Quantity	Cap color	Solution color	Description / Contents
Sample Buffer (5x)	1 x 20ml	White	Yellow	5 x concentrated Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Wash Buffer (50x)	1 x 20ml	White	Green	50 x concentrated Tris, NaCl, Tween 20, sodium azide < 0.1% (preservative)
<b>READY TO USE</b>				
Item	Quantity	Cap color	Solution color	Description / Contents
Cut-off Calibrator	1 x 1.5ml	Blue	Yellow	Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Calibrators A-D	4 x 1.5ml	White	Yellow *	Concentration of each calibrator: 0, 10, 30, 100 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative)
Conjugate, IgG	1 x 15ml	Blue	Blue	Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA)
TMB Substrate	1 x 15ml	Black	Colorless	Stabilized tetramethylbenzidine and hydrogen peroxide (TMB/H <sub>2</sub> O <sub>2</sub> )
Stop Solution	1 x 15ml	White	Colorless	1M Hydrochloric Acid
Microtiter plate	12 x 8 well strips	N/A	N/A	With breakaway microwells. Refer to paragraph 1 for coating.
* Color increasing with concentration				
<b>MATERIALS REQUIRED, BUT NOT PROVIDED</b>				
Microtiter plate reader 450 nm reading filter and recommended 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 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 at 2-8°C/35-46°F for at least 1 month. 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.

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## 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 the intended 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<sub>3</sub>) as a preservative. NaN<sub>3</sub> may be toxic if ingested or adsorbed by skin or eyes. NaN<sub>3</sub> 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.

The kit contains material of animal origin as stated in the table of contents, handle according to national requirements.

### 5.2 General directions for use

In case that the product information, including the labeling, is defective or incorrect please contact the manufacturer or the supplier of the test kit.

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.**

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## 6 Sample Collection, Handling and Storage

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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 during the first 8h, respectively stored tightly closed at 2-8°C/35-46°F up to 48h, or frozen at -20°C/-4°F for longer periods

## 7 Assay Procedure

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### 7.1 Preparations prior to starting

#### **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).

To avoid mistakes we suggest to mark the cap of the different calibrators.

#### **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 Pipetting Scheme

We suggest pipetting calibrators, controls and samples as follows:

Antigen		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							
		1	2	3	4	5	6	7	8	9	10	11	12
Cal. antigen	<b>A</b>	CalA	CalB	CalC	CalD			CalA	CC	CalD			
PR3	<b>B</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
MPO	<b>C</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
BPI	<b>D</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Elastase	<b>E</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Cathepsin G	<b>F</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Lysozym	<b>G</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Lactoferrin	<b>H</b>	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12

CalA: calibrator A

CalB: calibrator B

CalC: calibrator C

CalD: calibrator D

CalE: calibrator E

CalF: calibrator F

PC: positive control

NC: negative control



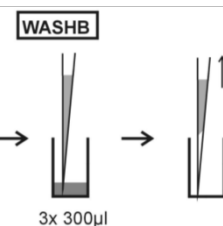
CC: cut-off calibrator


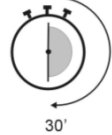
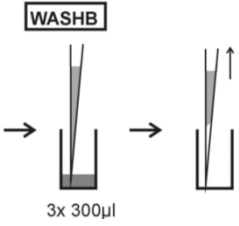

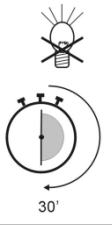
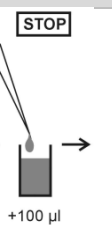

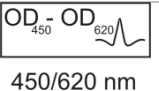
P1: patient 1

P2: patient 2

P3: patient 3

## 7.3 Test Steps

Step	Description
1.	Ensure preparations from step 7.1 above have been carried out prior to pipetting.
2.	Use the following steps in accordance with quantitative/ qualitative interpretation results desired:
<b>CONTROLS &amp; SAMPLES</b>	
3.	 <p>Pipette into the designated wells as described in chapter 7.2 above, 100 µl of either:</p> <ol style="list-style-type: none"> <li>Calibrators (CAL.A to CAL.D) for <i>QUANTITATIVE</i> or</li> <li>Cut-off Calibrator (CC) for <i>QUALITATIVE</i> interp.</li> </ol> <p>and 100 µl of each of the following:</p> <ul style="list-style-type: none"> <li>CalA and CalD, and</li> <li>Patients diluted serum (P1, P2...)</li> </ul>
4.	 <p>Incubate for 30 minutes at 20-32°C/68-89.6°F.</p>
5.	 <p>Wash 3x with 300 µl washing buffer (diluted 1:50).</p>

CONJUGATE	
6.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Pipette 100 µl conjugate into each well.</p> </div> </div>
7.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Incubate for 30 minutes at 20-32°C/68-89.6°F.</p> </div> </div>
8.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Wash 3x with 300 µl washing buffer (diluted 1:50).</p> </div> </div>
SUBSTRATE	
9.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Pipette 100 µl TMB substrate into each well.</p> </div> </div>
10.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Incubate for 30 minutes at 20-32°C/68-89.6°F, protected from intense light.</p> </div> </div>
STOP	
11.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Pipette 100 µl stop solution into each well, using the same order as pipetting the substrate.</p> </div> </div>
12.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Incubate 5 minutes minimum.</p> </div> </div>
13.	<p>Agitate plate carefully for 5 sec.</p>
14.	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">  </div> <div> <p>Read absorbance at 450 nm (recommended 450/620 nm) within 30 minutes.</p> </div> </div>



## 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.

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
<b>Cut-off calibrator</b>	
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.

In case that the values of the controls do not meet the criteria the test is invalid and has to be repeated.

The following technical issues should be verified: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, photometer, incubation conditions and washing methods.

If the items tested show aberrant values or any kind of deviation or that the validation criteria are not met without explicable cause please contact the manufacturer or the supplier of the test kit.

### Qualitative Calculation

Calculation of the ANCA-Pro 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.

<b>Negative:</b>	<b>OD patient</b>	<b>&lt;</b>	<b>0.8 x OD cut-off</b>
<b>Equivocal:</b>	<b>0.8 x OD cut-off</b>	<b>≤</b>	<b>OD patient ≤ 1.2 x OD cut-off</b>
<b>Positive:</b>	<b>OD patient</b>	<b>&gt;</b>	<b>1.2 x OD cut-off</b>

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## 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:	96 tests

## 10 Performance Data

### 10.1 Analytical sensitivity

Testing sample buffer 30 times on ANCA-Pro gave an analytical sensitivity 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.

### 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.

Sample No.	Dilution Factor	Measured (U/ml)	Expected (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

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## 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 ANCA-Pro is calibrated in arbitrary units (U/ml)

## 11 Literature

**Falk, RJ Jennette JC (1988).** Antineutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 318: 1651-1657.








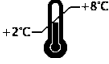












**Lüdemann J, Utecht B, Gross WL (1990).** Antineutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinophil enzyme. *J Exp Med* 171: 375-362.

**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.

**Gross WL, Hauschild S, Mistry N (1993)** .The clinical relevance of ANCA in vasculitis 7-11 5th International ANCA Workshop, Cambridge Clin Exp Immunol 93 (Suppl. 1).

**Peen E, Almer S, Bodemar G, Ryden BO, Sjolín 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.



	- 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
	- Numero d'ordine - Référence Catalogue - Bestellnummer - Número de catálogo	- Catalogue number - Numéro de catálogo - Αριθμός παραγγελίας
	- Descrizione lotto - Lot - Chargen Bezeichnung - Lote	- Lot - Lote - Χαρακτηρισμός παρτίδας
	- Conformità europea - Déclaration CE de Conformité - Europäische Konformität - Declaração CE de Conformidade	- EC Declaration of Conformity - Declaración CE de Conformidad - Ευρωπαϊκή συμφωνία
	- 96 determinazioni - 96 tests - 96 Bestimmungen - 96 Testes	- 96 tests - 96 pruebas - 96 προσδιορισμοί
	- Rispettare le istruzioni per l'uso - Voir les instructions d'utilisation - Gebrauchsanweisung beachten - Ver as instruções 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
	- 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
	- Calibratore cut-off - Etalon Seuil - Grenzwert Kalibrator - Calibrador de cut-off	- Cut off Calibrator - Calibrador de cut-off - Οριακός ορός Αντιδραστήριο βαθμονόμησης
	- Controllo positivo - Contrôle Positif - Positiv Kontrolle - Controllo positivo	- Positive Control - Control Positivo - Θετικός ορός ελέγχου
	- Controllo negativo - Contrôle Négatif - Negativ Kontrolle - Controllo negativo	- Negative Control - Control Negativo - Αρνητικός ορός ελέγχου
	- Calibratore - Etalon - Kalibrator - Calibrador	- Calibrator - Calibrador - Αντιδραστήριο βαθμονόμησης
	- Recupero - Corrélation - Wiederfindung - Recuperação	- Recovery - Recuperado - Ανάκτηση
	- Coniugato - Conjugé - Konjugat - Conjugado	- Conjugate - Conjugado - Σύζευγμα
	- Micropiastra rivestita - Microplaque sensibilisée - Beschichtete Mikrotiterplatte - Microplaca revestida	- Coated microtiter plate - Microplaca sensibilizada - Επικαλυμμένη μικροπλάκα
	- Tampone di lavaggio - Tampon de Lavage - Waschpuffer - Solução de lavagem	- Wash buffer - Solución de lavado - Ρυθμιστικό διάλυμα πλύσης
	- Tampone substrato - Substrat - Substratpuffer - Substrato	- Substrate buffer - Tampón sustrato - Ρυθμιστικό διάλυμα υποστρώματος
	- Reagente bloccante - Solution d'Arrêt - Stopreagenz - Solução de paragem	- Stop solution - Solución de parada - Αντιδραστήριο διακοπής αντίδρασης
	- Tampone campione - Tampon Echantillons - Probenpuffer - Diluente de amostra	- Sample buffer - Tampón Muestras - Ρυθμιστικό διάλυμα δειγμάτων