

*AESKULISA* ENA-6Pro

REF 3103

# Instruction manual

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## 1. Intended Use

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**AESKULISA ENA-6Pro** is a solid phase enzyme immunoassay for the separate semi-quantitative detection of IgG antibodies against six cellular and nuclear antigens in human serum. The wells are coated with recombinant SS-B, SS-A 52 kDa, Scl 70, Jo-1 and highly purified native human snRNP/Sm, Sm and SS-A 60 kDa. The assay is a tool in the differential diagnosis of systemic rheumatic diseases.

## 2. Clinical Application and Principle of the Assay

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Anti-nuclear antibodies (ANA) are an important tool for the differential diagnosis of systemic rheumatic diseases, Indirect immunofluorescence test (IFT) on eucaryotic cells like HeLa has been the established method for the detection of ANAs. Single antibody specificities are distinguished by fluorescence patterns but more specific testing by ELISAs employing the target antigens are available too for a simple and reliable differentiation of ANAs.

ANAs are especially found in active and inactive systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD), scleroderma, Sjögren`s syndrome, polymyositis.

### **ANA antibodies against:**

-Sm (Smith antigen) are directed against core proteins (B,B', D1-D3, E, F, G) of small nuclear ribonucleoproteins (snRNPs). Anti-Sm as well as antibodies against double stranded DNA (dsDNA) are highly specific for SLE and thus are included in diagnostic and classification criteria for SLE.

-snRNP/Sm complex are directed against Sm and U1 snRNP proteins (70 kDa, A and C). They occur in SLE, Sjögren`s syndrome, scleroderma and polymyositis.

-SS-A (Ro; soluble cytoplasmic and/or nuclear ribonucleoproteins of 52 kDa and 60 kDa) and antibodies against SS-B (La; 48 kDa protein associated with RNA polymerase III) are mainly found in high titers for primary and secondary Sjögren`s syndrome but also in SLE, congenital heartblock and neonatal lupus.

-Scl-70 are directed against DNA-topoisomerase I. They are highly specific for systemic scleroderma and give a hint for a severe course.

-Jo-1 are directed against histidyl-tRNA synthetase (cytoplasmic protein involved in protein biosynthesis) and are found in 20-40 % of patients with polymyositis and dermatomyositis.

### ***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

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#### **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, 1.5 ml each 0, 10, 30, 100 U/ml (capped white: 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<sub>2</sub>O<sub>2</sub>

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

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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

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### 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 ( $\text{NaN}_3$ ) as a preservative.  $\text{NaN}_3$  may be toxic if ingested or adsorbed by skin or eyes.  $\text{NaN}_3$  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

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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 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

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### 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 antigen coating of wells see Annex A2, for 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 of calibrators or cut-off calibrator 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

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	0.042
10 U/ml	0.323
30 U/ml	0.757
100 U/ml	1.602
<b>Cut-off calibrator</b>	
15 U/ml	0.451 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 ENA-6Pro* 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:**  $OD_{\text{patient}} < 0.8 \times OD_{\text{cut-off}}$

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

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

## 9. Technical Data

<b>Sample material:</b>	serum
<b>Sample volume:</b>	10 µl of sample diluted 1:101 with 1x sample buffer
<b>Total incubation time:</b>	90 minutes at 20-32°C/68-89.6°F
<b>Calibration range:</b>	0-100 U/ml
<b>Analytical sensitivity:</b>	1.0 U/ml
<b>Storage:</b>	at 2-8°C/35-46°F use original vials, only
<b>Number of determinations:</b>	96 tests

## 10. Performance Data

### 10.1 Analytical sensitivity

Testing sample buffer 30 times on *AESKULISA ENA-6Pro (REF7103)* gave an analytical sensitivity of 1.0 U/ml.

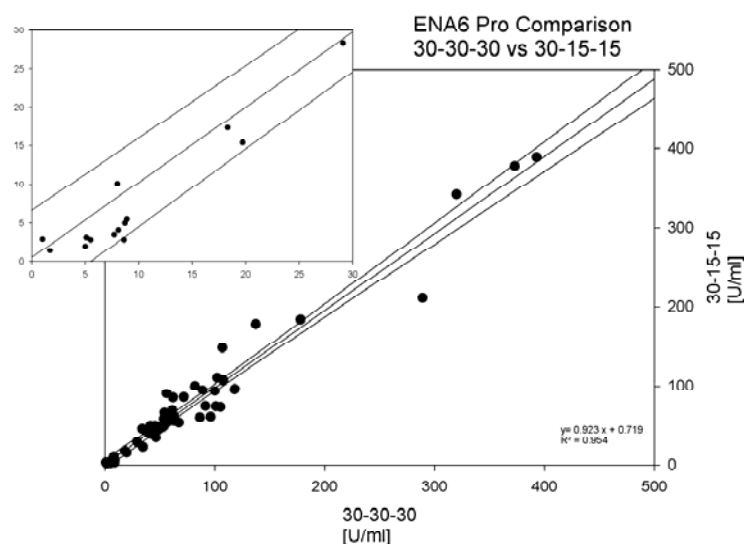
### 10.2 Specificity and sensitivity

The microplate is coated with highly purified and/or recombinant antigens (SS-A, SS-B, snRNP/Sm, Sm, Scl-70, Jo-1). No crossreactivities to other autoantigens have been found. The data has been acquired with the *AESKULISA ENA-6Pro (REF7103)*.

### Correlation:

The comparability of performance data was assessed with 62 sera tested on both, *AESKULISA 7103* and *AESKULISA 3103*. A linear regression analysis of the two products showed that the two products are equivalent. Included in these sera are 15 sera close to cut-off.

	Sensitivity
SS-A	80% for Sjögren's syndrome
SS-B	40-74% for Sjögren's syndrome
Sm	10-30% for SLE
U1-snRNP	100 % for mixed connective tissue disease
Scl 70	20-48% for systemic scleroderma
Jo-1	25% for polymyositis and dermatomyositis





### 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. Scl-70	Dilution Factor	measured concentration (U/ml)	expected concentration (U/ml)	Recovery (%)
1	1 / 100	112.0	110.0	101.8
	1 / 200	56.4	55.0	102.6
	1 / 400	28.0	27.5	101.8
	1 / 800	14.3	13.8	103.6
2	1 / 100	83.8	85.0	98.6
	1 / 200	41.1	42.5	96.7
	1 / 400	20.8	21.3	97.7
	1 / 800	9.8	10.6	92.5

### 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		
ENA-6Pro	Mean U/ml	CV (%)
SSA	45.7	1.5
SSB	124.8	2.6
SnRNP	20.0	3.1
Sm	51.6	1.7
Scl-70	19.3	3.1
Jo-1	65.5	4.2

Inter-Assay		
ENA-6Pro	Mean U/ml	CV (%)
SSA	44.2	1.3
SSB	123.3	2.4
SnRNP	21.7	2.8
Sm	54.6	3.9
Scl-70	22.4	3.7
Jo-1	68.4	1.7

### 10.5 Calibration

The *AESKULISA ENA-6Pro* is calibrated against reference sera from the CDC (Centers for Disease Control and Prevention) Atlanta.

## 11. Literature

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- 1. Antinuclear antibody.**  
The Lancet 1984, Sept. 15: 611-13.
- 2. Froelich CH, Wallmann H, Skosey JL and Teodorescu M.**  
*Clinical value of an integrated ELISA system for the detection of 6 autoantibodies.*  
The Journal of Rheumatology 1990; 17 (2): 192-200.
- 3. Mierau R, Genth E.**  
*Autoantikörper bei systemischem Lupus erythematodes und verwandten Erkrankungen*  
In: Thomas L. (Hrsg.) Labor und Diagnose.  
TH-Books, Frankfurt, 1998, 5. Auflage: 843-851.
- 4. Schmolke M, Oppermann M, Helmke K, Guder WG.**  
*Antibody determination against ENA- a challenge for the routine laboratory.*  
Poster P59, 5 th Dresden Symposium on Autoantibodies, 2000.

## 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 <b>quantitative interpretation</b> use calibrators to establish a standard curve						for <b>qualitative interpretation</b> 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	<b>A</b>	CalA	CalB	CalC	CalD			CalA	CC	CalD			
Cal antigen	<b>B</b>	CalA	CalB	CalC	CalD			CalA	CC	CalD			
SS-A	<b>C</b>	P1	P2	P3	P4	P5	...	P1	P2	P3	P4	P5	...
SS-B	<b>D</b>	P1	P2	P3	P4	P5	....	P1	P2	P3	P4	P5	....
Sm	<b>E</b>	P1	P2	P3	P4	P5	...	P1	P2	P3	P4	P5	...
snRNP/Sm	<b>F</b>	P1	P2	P3	P4	P5	...	P1	P2	P3	P4	P5	...
Scl70	<b>G</b>	P1	P2	P3	P4	P5	...	P1	P2	P3	P4	P5	...
Jo-1	<b>H</b>	P1	P2	P3	P4	P5	...	P1	P2	P3	P4	P5	...

CalA: Calibrator A, CalB: Calibrator B, CalC: Calibrator C, CalD: Calibrator D

CC: Cut-off calibrator

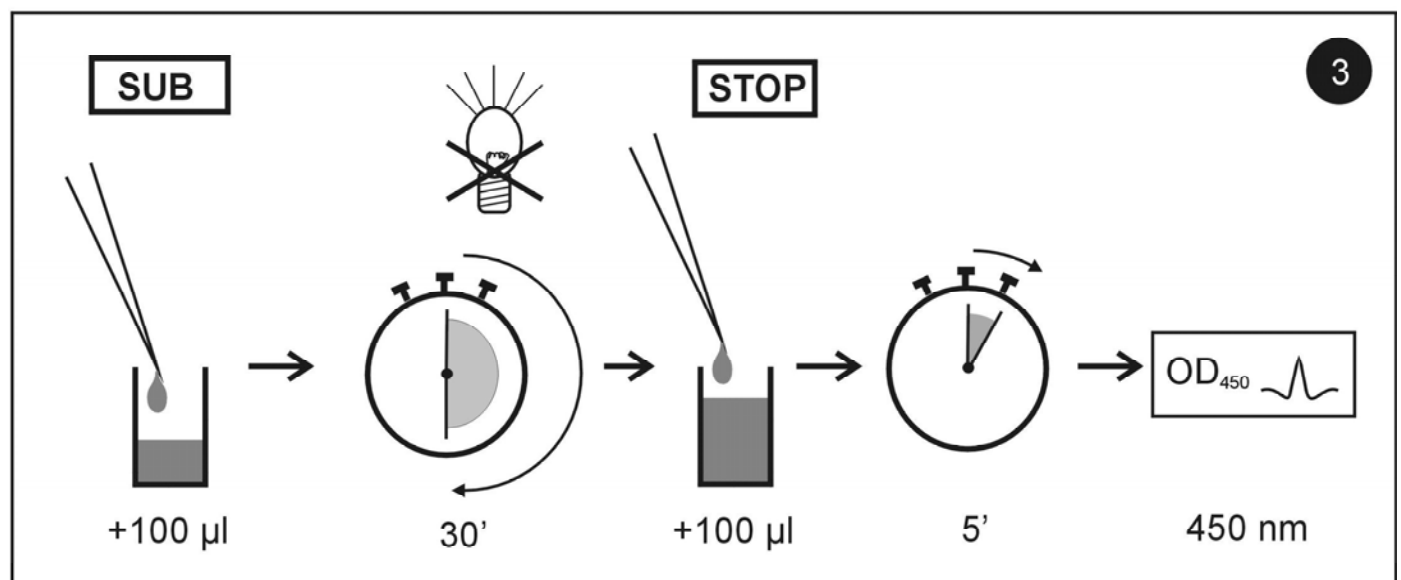
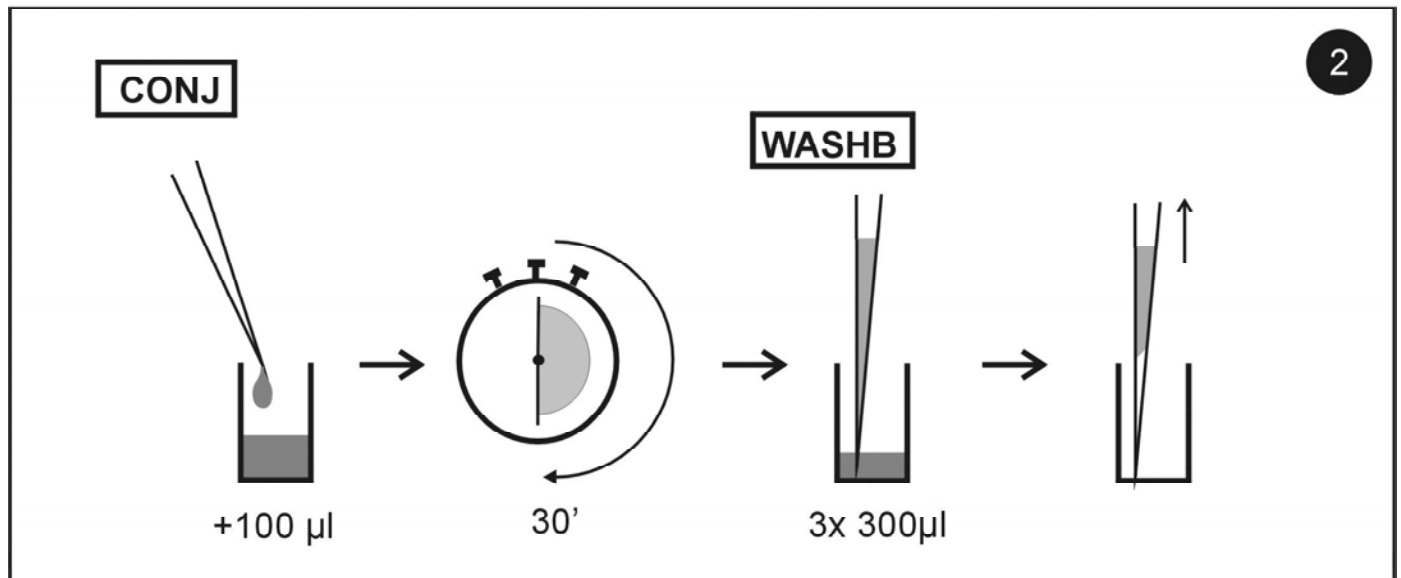
P1: patient 1

P2: patient 2

P3: patient 3

Cal antigen: antigen for calibrators

## Annex B: Test Procedure

























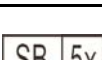
Assay/Test: \_\_\_\_\_ Incubation / Inkub. : 1. \_\_\_\_\_ min Date/ Datum: \_\_\_\_\_

Temperature/Temperatur: \_\_\_\_\_ °F \_\_\_\_\_ °C 2. \_\_\_\_\_ min

Signature/Unterschrift: \_\_\_\_\_

Name: \_\_\_\_\_ 3. \_\_\_\_\_ min

	1	2	3	4	5	6	7	8	9	10	11	12
A	calibrator A	calibrator B	calibrator C	calibrator D	or alternative for	cut off calibrator						
B	calibrator 0 U /ml	calibrator 10 U /ml	calibrator 30 U /ml	calibrator 100 U /ml	qualitative interpretations	cut off calibrator						
SSA 52/60												
SSB												
Sm												
SmSNP												
Scl-70												
Jo-1												

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	<ul style="list-style-type: none"> <li>◆ Da utilizzarsi entro</li> <li>◆ Utilise avant le</li> <li>◆ Verwendbar bis</li> <li>◆ Utilizar antes de</li> </ul>	<ul style="list-style-type: none"> <li>◆ Use by</li> <li>◆ Utilizar antes de</li> <li>◆ Χρήση μέχρι</li> </ul>
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	<ul style="list-style-type: none"> <li>◆ Coniugato</li> <li>◆ Conjugé</li> <li>◆ Konjugat</li> <li>◆ Conjugado</li> </ul>	<ul style="list-style-type: none"> <li>◆ Conjugate</li> <li>◆ Conjugado</li> <li>◆ Σύζευγμα</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Micropiastra rivestita</li> <li>◆ Microplaque sensibilisée</li> <li>◆ Beschichtete Mikrotiterplatte</li> <li>◆ Microplaca revestida</li> </ul>	<ul style="list-style-type: none"> <li>◆ Coated microtiter plate</li> <li>◆ Microplaca sensibilizada</li> <li>◆ Επικαλυμμένη μικροπλάκα</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Piastra ad aghi rivestita</li> <li>◆ Pinplate sensibilisée</li> <li>◆ Beschichtete Pinplatte</li> <li>◆ Pinplate revestida</li> </ul>	<ul style="list-style-type: none"> <li>◆ Coated pinplate</li> <li>◆ Pinplate sensibilizada</li> <li>◆ Επικαλυμμένη πλάκα Pin</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Tampone di lavaggio</li> <li>◆ Tampon de Lavage</li> <li>◆ Waschpuffer</li> <li>◆ Solução de lavagem</li> </ul>	<ul style="list-style-type: none"> <li>◆ Wash buffer</li> <li>◆ Solución de lavado</li> <li>◆ Ρυθμιστικό διάλυμα πλύσης</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Tampone substrato</li> <li>◆ Substrat</li> <li>◆ Substratpuffer</li> <li>◆ Substrato</li> </ul>	<ul style="list-style-type: none"> <li>◆ Substrate buffer</li> <li>◆ Tampón sustrato</li> <li>◆ Ρυθμιστικό διάλυμα υποστρώματος</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Reagente bloccante</li> <li>◆ Solution d'Arrêt</li> <li>◆ Stopreagenz</li> <li>◆ Solução de paragem</li> </ul>	<ul style="list-style-type: none"> <li>◆ Stop solution</li> <li>◆ Solución de parada</li> <li>◆ Αντιδραστήριο διακοπής αντίδρασης</li> </ul>
	<ul style="list-style-type: none"> <li>◆ Tampone campione</li> <li>◆ Tampon Echantillons</li> <li>◆ Probenpuffer</li> <li>◆ Diluente de amostra</li> </ul>	<ul style="list-style-type: none"> <li>◆ Sample buffer</li> <li>◆ Tampón Muestras</li> <li>◆ Ρυθμιστικό διάλυμα δειγμάτων</li> </ul>