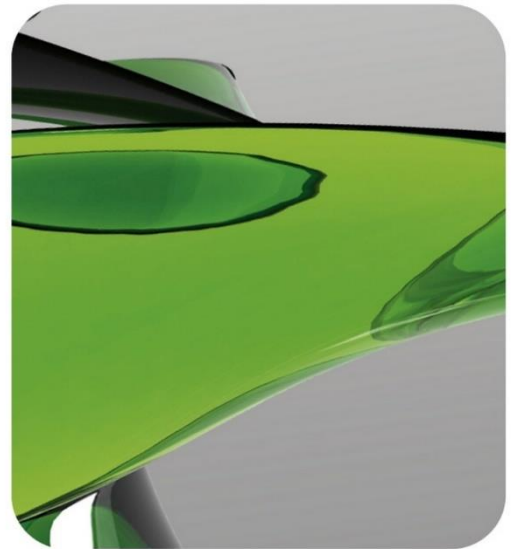




AESKULISA[®]

THE DIAGNOSTIC TOOL THAT WORKS

INSTRUCTION MANUAL

AESKULISA[®] PR3

REF 30-7302US





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Instruction manual

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1 Intended Use

AESKULISA® PR3 is a solid phase enzyme immunoassay employing highly purified native human proteinase 3 (PR3) from human neutrophil granulocytes for the semiquantitative and qualitative detection of antibodies against proteinase 3 in human serum.

The assay is an aid in the diagnosis of Wegener's granulomatosis and should be used in conjunction with other serological tests and clinical findings.

2 Clinical Application and Principle of the Assay

Antibodies against proteinase 3 (PR3) belong to the group of anti-neutrophil cytoplasmic antibodies (ANCA) which are directed against cytoplasmic components of neutrophilic granulocytes and monocytes.¹ Indirect immunofluorescence test (IFT) on ethanol-fixed neutrophils has been the established method for the detection of ANCAs. It became apparent that some ANCAs create a cytoplasmic fluorescence pattern (thus called cANCA) while others create a perinuclear pattern (the pANCA).^{2,3} As both patterns may cover multiple antigens, immunofluorescence is not suitable for a satisfying differential diagnosis of vasculitis; thus, each IFT should be verified with specific ELISA tests.³

PR3 is a serine protease from the azurophile granules of neutrophils with a molecular weight of 29 kDa.^{5,6} The cationic protein has a proteolytic activity to elastin.^{5,6} different matrix components such as laminin, vitronectin, fibronectin and collagen type IV⁷ and other proteins such as casein and hemoglobin.⁵

ANCA are important markers for the differential diagnosis of autoimmune vasculitis. Autoantibodies to PR3 are a specific serological marker for Wegener's granulomatosis (WG) with a high occurrence (up to 95%) depending on disease activity.^{8,9} Even though the etiology and pathogenesis is still obscure, autoantibodies to PR3 are thought to play an active role in the pathogenesis of WG.³ Importantly anti-PR3 titers are strongly associated with disease activity.⁹

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:

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 IgG (capped blue: blue solution)
Containing: Goat Anti-human IgG conjugated to horseradish peroxidase, BSA, PBS

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 purified native human proteinase 3

Material required but not provided:

Microtiter plate reader 450 nm reading filter and optional 620 nm filter (600-690 nm). Glass ware (cylinder 100-1000 ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 µl) or adjustable multipipette (100-1000 µl). 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 and the European Pharmacopeia.

4 Storage and Shelf Life

Store all reagents and the microplate at 2-8°C/35.6-46.4°F, in their original containers. Once prepared, reconstituted solutions are stable for 1 month at 2-8°C/35.6-46.4°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 the 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 FDA 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 ($20\text{--}32^\circ\text{C}/68\text{--}89.6^\circ\text{F}$) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Never expose components to higher temperature than $37^\circ\text{C}/98.6^\circ\text{F}$.

Always pipette substrate solution with brand new tips only. Protect this reagent from light. Never pipette conjugate with tips used with other reagents prior.

5.3 Limitations

The test performance has not been established for the pediatric population. 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 and medicinal 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 during the first 8h, respectively stored tightly closed at $2\text{--}8^\circ\text{C}/35.6\text{--}46.4^\circ\text{F}$ up to 48h, or frozen at $-20^\circ\text{C}/-4^\circ\text{F}$ for longer periods (according CLSI Guideline H18-A3).

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/35.6-46.4°F)

7.2 Work flow

For pipetting scheme see Annex A, for the test procedure see Annex B

We recommend pipetting 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 control and negative and positive controls into the designated wells.
- Incubate for 30 minutes at room temperature (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 room temperature (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 room temperature (20-32°C/68-89.6°F).
- 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 Semiquantitative and Qualitative 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**.

Normal Range	Positive Results
≤ 15 U/ml	> 15 U/ml

Example of a standard curve

We recommend pipetting calibrators in parallel for each run.

Calibrators IgG	OD 450/620 nm	CV % (Variation)
0 U/ml	0.040	0.7
3 U/ml	0.175	2.7
10 U/ml	0.350	1.8
30 U/ml	0.680	3.4
100 U/ml	1.220	0.4
300 U/ml	2.130	0.8

Example of calculation

Patient	OD 450/620 nm	Result (U/ml)
P 01	0.565	23.0
P 02	1.022	63.8

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 national 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's OD with the OD of the cut-off calibrator. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

Samples above the calibrator range should be reported as > 300 U/ml. They should be diluted as appropriate and reassayed. Samples below calibrator range should be reported as < Min.

Negative: $OD_{\text{patient}} \leq OD_{\text{cut-off}}$

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

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:	0-300 U/ml
Analytical sensitivity:	1.38 U/ml
Storage:	at 2-8°C/35.6-46.4°F use original vials, only
Number of determinations:	96 tests

10 Performance Data

10.1 Limit of Detection

Testing sample buffer 30 times on **AESKULISA® PR3** and 8 low negative samples for 8 times gave a limit of detection of 1.38 U/ml.

10.2 Specificity and Sensitivity

The microtiter plates are coated with highly **purified native human proteinase 3** from human neutrophil granulocytes. No cross-reactivity to other autoantigens has been found. The diagnostic specificity of PR3 antibodies for active Wegener's Granulomatosis is 70-80% and 60% in limited Wegener's Granulomatosis.³

149 sera of patients suffering from Wegener's Granulomatosis, microscopic polyangiitis and other autoimmune diseases have been tested on the **AESKULISA® PR3** and a predicate device, of these 69 sera lay in range of the assay and were used for a comparison study versus a predicate device.

* An equivocal results of the predicate device have been considered as a negative for this calculation.

Overall percent agreement*	84.1%	73.7% to 90.9%
Positive percent agreement	100%	79.6% to 100%
Negative percent agreement*	79.6%	67.1% to 88.2%

		predicate device			Total
		pos	equiv	neg	
AESKULISA® PR3	pos	15	6	5	26
	neg	0	4	39	43
	Total	15	10	44	69



For a clinical comparison study only Wegener's Granulomatosis samples were considered as positive for the diagnostic sensitivity/specificity calculation, all other diagnosis, though there may be PR3 antibodies present were considered as "to be negative" (complete data upon request).

		95% C.I.
Overall percent agreement	91.4 %	85.5% to 95.0%
diagnostic sensitivity	100%	93.7% to 100%
diagnostic specificity	85.4%	76.1% to 91.4%

		diagnosis		
		pos	neg	Total
AESKULISA® PR3	pos	57	12	69
	neg	0	70	70
	Total	57	82	139

Number of samples with	AESKULISA® PR3		
	pos	neg	Total
acute hearing loss	1	0	1
chronic renal disease	1	0	1
Churg-Strauss	1	1	2
COPD	0	3	3
Crohn's disease	0	6	6
endocarditis	1	0	1
glomerulo nephritis	0	2	2
Goodpasture-Syndrome	1	0	1
healthy	0	1	1
HIV	1	0	1
mPAN	0	30	30
palsy	1	0	1
Polymyalgia rheumatica (vasculitis)	1	0	1
Reactive Arthritis	0	21	21
rheumatoid arthritis	1	0	1
SLE	1	0	1
ulcerative colitis	0	6	6
ulcerative colitis, septic fungal infection	1	0	1
glomerulonephritis (c-ANCA pos)	1	0	1
Wegener's Granulomatosis	56	0	56
Wegener's Granulomatosis / GN	1	0	1
Total	69	70	139



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 (U/ml)	expected (U/ml)	Recovery 90-110%
1	1 / 100	102.0	105.0	97.1
	1 / 200	49.8	52.5	94.9
	1 / 400	25.8	26.3	98.1
	1 / 800	12.1	13.1	92.4
2	1 / 100	52.6	54.0	96.3
	1 / 200	25.8	27.0	95.5
	1 / 400	14.1	13.5	104.4
	1 / 800	7.3	6.8	107.4

Sample No.	Dilution Factor	measured (U/ml)	expected (U/ml)	Recovery 90-110%
3	1 / 100	278.4	261.8	106.3
	1 / 200	139.5	130.9	106.6
	1 / 400	64.2	65.4	98.2
	1 / 800	32.3	32.7	98.8
4	1 / 100	33.6	34.2	98.2
	1 / 200	16.0	17.1	93.6
	1 / 400	7.9	8.6	91.9
	1 / 800	4.2	4.3	97.7

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. The accepted range for CV is 10% for positive samples, 15% around cut-off and 20% for negative samples (N=40 / 40 / 24).

Intra-Assay		
Sample No.	Mean (U/ml)	CV (%)
1	6.8	7.1
2	16.3	3.4
3	11.1	2.0
4	75.4	3.4
5	260.7	4.4
6	22.9	2.2
7	48.9	3.7
8	88.2	5.2

Inter-Assay		
Sample No.	Mean (U/ml)	CV (%)
1	6.8	15.8
2	16.3	6.4
3	11.1	4.7
4	75.4	6.0
5	260.7	6.0
6	22.9	2.2
7	48.9	4.0
8	88.2	5.7

Lot-to-Lot variability		
Sample No.	Mean (U/ml)	CV (%)
1	5.8	12.8
2	16.2	6.5
3	11.0	6.5
4	74.9	3.1
5	263.8	5.7
6	22.3	1.7
7	51.2	2.8
8	100.0	8.7

10.5 Calibration

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



11 Literature

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12 Regulatory Symbols

IVD	For in vitro diagnostic use
REF	Catalog number
LOT	Lot
UDI	Unique Device Identifier
	96 tests
	See instructions for use
	Use by
	Store at 2-8°C (35.6-46.4°F)
	Manufactured by
CON +	Positive Control
CON -	Negative Control
CAL	Calibrator
CO-CAL	Cut off Calibrator
CONJ	Conjugate
MP	Coated microtiter plate
WASHB 50x	Wash buffer
SUB	Substrate buffer
STOP	Stop solution
SB 5x	Sample buffer
Rx only	For Prescription Use only



Annex

A: Pipetting scheme

We suggest pipetting calibrators, controls and samples as follows:

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

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

	for semiquantitative interpretation use calibrators to establish a standard curve						for qualitative interpretation use cut-off calibrator					
	1	2	3	4	5	6	7	8	9	10	11	12
A	CalA	CalE	P1				NC	P3				
B	CalA	CalE	P2				NC	P4				
C	CalB	CalF	P3				CC	P5				
D	CalB	CalF	P4				CC	P6				
E	CalC	PC	P5				PC	...				
F	CalC	PC	P6				PC	...				
G	CalD	NC	...				P1	...				
H	CalD	NC	...				P2	...				

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

B: Test Procedure

