Version 08-11/24

User's Manual

Anti-Pseudomonas aeruginosa ELISA Kit

REF **DEIA-NB24-22**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Anti-Pseudomonas aeruginosa ELISA Kit is an enzyme immunoassay for qualitative and quantitative detection of IgG-Antibodies in human serum against the extracellular proteins: Alkaline Protease (AP), Elastase (ELA) and Exotoxin A (Exo A) of Pseudomonas aeruginosa.

General Description

Pseudomonas aeruginosa, a Gram-negative bacterium ubiquitously distributed in the moist environment, causes about 10% of all nosocomial infections. This opportunistic pathogen leads to acute and chronic types of infection within various organs of susceptible patient groups. Chronic pulmonary infection in patients with cystic fibrosis (CF) is very frequent, may start early in infancy and determines the life expectancy of these patients. P. aeruginosa infection provokes a production of antibodies to a large number of P. aeruginosa antigens in CF patients. Creative Diagnostics's sensitive antibody detection system is an aid to discriminate between infected and uninfected patient groups.

Depending on the *Pseudomonas aeruginosa* species and the patient's immune reaction, antibodies can be detected against a single, two or even all three antigens simultaneously. A patient is regarded as sero-positive when the serum is positive for one or more of the antigens.

Used for research purposes and cannot be used as a diagnostic basis.

Principles of Testing

The Anti-Pseudomonas aeruginosa ELISA Kit is a sandwich enzyme immunoassay. Serum or plasma samples are diluted and added to the wells of a microtiter plate, which have been previously coated with the Pseudomonas aeruginosa antigens alkaline protease, elastase or exotoxin A. Specific antibodies in the sample bind to the respective antigens during an incubation of 2 h at 37°C. After washing, the conjugate (anti-human IgG peroxidase-labelled immunoglobulin) is added and incubated again 2 h at 37°C. After a final washing step, substrate is added and incubated for 30 min at room temperature. The color forming reaction is terminated on addition of stop solution accompanied by a color change from blue to yellow. The absorbance of the coloured reaction product is measured on a microtiter plate reader. The colour intensity of the reaction corresponds to the concentration of antibodies in the sample.

Reagents And Materials Provided

1. **Microtiter plate 1 (AP):** ready for use, coated with alkaline Protease, labelled red. Wells are separately breakable. (8x12) wells
2. **Microtiter plate 2 (ELA):** ready for use, coated with Elastase, labelled blue. Wells are separately breakable. (8x12) wells
3. **Microtiter plate 3 (Exo A):** ready for use, coated with Exotoxin A, labelled green. Wells are separately breakable. (8x12) wells
4. **Positive Control 1 (PC1):**, ready for use, calibrated control serum for alkaline Protease, AP, red labelled, shows a titer of 1:2500. 1 x 1.5 mL

5. **Positive Control 2 (PC2):**, ready for use, calibrated control serum for Elastase, ELA, blue labelled, shows a titer of 1:2500. 1 x 1.5 mL
6. **Positive Control 3 (PC3):**, ready for use, calibrated control serum for Exotoxin A, Exo A, green labelled, shows a titer of 1:2500. 1 x 1.5 mL
7. **Negative Control (NC):** ready for use, contains human serum, not reactive for Pseudomonas aeruginosa antigens. 2 x 1.5 mL
8. **Control (CTR):** ready for use, contains human serum and has to be determinated as borderline for Pseudomonas aeruginosa antigens in the assay 2 x 1.5 mL
9. **Antibody-HRP-Conjugate (DET):** 100-fold concentrated, contains horseradish peroxidase (HRP)-labelled anti-human IgG. 2 x 250 µL
10. **Dilution Buffer (DIL):** ready for use. Please shake before use! 3 x 50 mL
11. **Washing Buffer (WB):** 20-fold concentrated solution. 1 x 120 mL
12. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 33 mL
13. **Stopping Solution (STP):** ready for use, 0.2 M sulfuric acid. 1 x 33 mL
14. **Sealing Tape:** for covering the microtiter plate. 6

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WP (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Vortex-mixer
5. Microtiter plate washer (recommended)
6. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
7. Incubator 37°C

Storage

Store the kit at 2-8°C after receipt until its expiry date.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided.

Specimen Collection And Preparation

1. Sample type: Serum and EDTA-Plasma samples

2. Specimen collection

Use standard venipuncture for the blood sampling. Haemolytic reactions are to be avoided.

3. Required sample volume: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: at least 2 days

• Storage at -20°C: at least 2 years

• Freeze-thaw cycles max. 3

After the separation of coagulated and / or corpuscular components, it is recommended to store the samples as soon as possible at -20°C and to avoid freeze-thaw cycles.

5. Sample dilution

Samples should be diluted in PE/PP-tubes. For larger sample numbers usage of a multi-stepper is recommended. If the signal of the sample exceeds the signal of the positive control (PC), the measurement must be repeated for an exact titer result with a higher diluted sample e.g. in three dilutions (1:1000; 1:10000 and 1:100000).

• Recommended dilution: 1:1000 with Dilution Buffer DIL.

• Exemplary Dilution Protocol

1:10 Pre-Dilution——90 µL Dilution Buffer DIL + 10 µL Serum- oder EDTA-Plasma

The pre-diluted sample should then be diluted as follows:

1:1000 Dilution——Add 10 µL of the 1:10 pre-diluted sample to 990 µL Dilution Buffer DIL

1:10000 Dilution——Add 100 µL of the 1:1000 dilution to 900 µL Dilution Buffer DIL

1:100000 Dilution——Add 100 µL of ther 1:10000 dilution to 900 µL Dilution Buffer DIL

• After mixing use 100 µL each of the needed sample dilution per well within 1 hour (Pipetting control = orange color of the solution in the microtiter plate well).

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. Use the Dilution Buffer DIL for the 1:100 dilution of Antibody-HRP-Conjugate DET. Please dilute only according to daily requirements.
2. The required volume of Washing Buffer WB is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest. The 1:20 diluted Washing Buffer WB is stable 4 weeks at 2-8°C. Please dilute only according to daily requirements.

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C.

   Sample Incubation: 2 h at 37°C, Conjugate Incubation 2 h at 37°C, Substrate Incubation: 30 min at room temperature 20°C - 25°C. The Substrate S, stabilised Tetramethylbencidine, is photosensitive: storage and incubation in the dark.
2. When performing the assay, Controls CTR, PC, NC and the samples should be pipette as fast as possible

(e.g. <15 minutes). To avoid distortions due to differences in incubation times, the diluted Antibody-HRP-Conjugate DET and the Substrate S should be added to the plate in the same order and in the same time interval as the samples. Stop Solution STP should be added to the plate in the same order as the Substrate S.

3. Washing: Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems. All washing must be performed with the provided Washing Buffer WB diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least. The danger of handling with potentially infectious material must be taken into account.

   Automatic washing: When using an Automatic microtiter plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

   Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Assay Step

1. Pipette in positions A1/2 of Plate 1 AP, Plate 2 ELA and Plate 3 Exo A 100 µl Negative Control NC.
2. Pipette in positions B1/B2 of Plate 1 AP 100 µl Positive Control PC1.

   Pipette in positions B1/B2 of Plate 2 ELA 100 µl Positive Control PC2.

   Pipette in positions B1/B2 of Plate 3 Exo A 100 µl Positive Control PC3.
3. Pipette in positions C1/2 of Plate 1 AP, Plate 2 ELA and Plate 3 Exo A 100 µl of the Control CTR.
4. Add 100 µl Sample Dilution SPE in the rest of the wells according to requirements.
5. Cover the wells with sealing tape and incubate the plate for 2 hours at 37°C.
6. After incubation aspirate the contents of the wells and wash the wells 3 times 300 µl Washing Buffer WB / well.
7. Following the last washing step pipette 100 µl of the diluted Antibody-HRP-Conjugate in each well.
8. Cover the wells with sealing tape and incubate the plate for 2 hours at 37°C.
9. After incubation wash the wells 3 times with Washing Buffer WB as described in step 6.
10. Pipette 100 µl of the Substrate Solution S in each well.
11. Incubate the microtiter plate for 30 minutes in the dark at room temperature (20°C -25°C).
12. Stop the reaction by adding 100 µl Stopping Solution STP to all wells.
13. Measure the absorbance within 30 minutes at 450 nm (Reference filter ≥ 590 nm).

Quality Control

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The Kit-control must be found within the acceptable range, which has been stated on the QC Certificate. The test results are only valid, if the test has been performed following the instructions. Moreover, the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws.

Quality criteria

For the evaluation of the assay it is preconditioned that the absorbance values of the Negative Controls NC should be below 0.25. The difference between the extinctions of Negative Controls NC and the respective Positive Control PC must be at least 0.6.

Calculation

Qualitative Calculation

The negative control (Blank) average is subtracted from the controls and samples to obtain absolute values.

The cut-off value is 20% of the absorbance of the Positive Control PC.

1: 1000 diluted samples with extinction values less than the cut off value are classified as negative.

The antibody titre is below 1:500.

1: 1000 diluted samples, having an absorbance in the range of ≥ 20% but < 50% of positive control, cannot be clearly classified and must therefore be assessed as borderline.

1: 1000 diluted samples, which extinctions are ≥ 50% of PC extinction, are to be classified as positive.

Exemplary qualitative calculation:

Negative Control NC	Extinction
1.Value	0.041
2.Value	0.056
Mean	**0.049**

Positive Control PC	Extinction
1.Value	1.120
2.Value	1.136
Mean	**1.128**

Calculation:

PC – NC : 1.128 – 0.049 = 1.079

Cut-off (20% of PC-NC) : 0.2 × 1.079 = 0.216

Borderline (50% of PC-NC) : 0.5 × 1.079 = 0.540

All samples with an extinction < 0.216 are determined as negative for Anti-P. aeruginosa IgG. Samples with an extinction of > 0.216 and < 0.540 are judged as borderline and samples showing an extinction of > 0.540 are determined as positive for the content of Anti-P. aeruginosa IgG.

Quantitative Calculation

The evaluation is carried out graphically or via an appropriate evaluation program. The extinction values of the negative NC and positive PC controls are plotted on the y-axis in a double-linear coordinate system against a titer factor. NC = 0 and PC = 2.5 on the x-axis. For the quantification of the sera values, a straight line is drawn

through the NC and PC values and extended to a titer factor of 3.5.

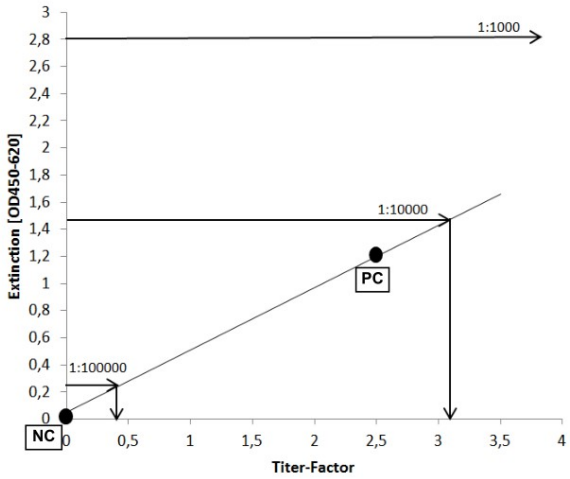
The titre of the individual serum is determined by reading the titre factor of the measured extinction value through the NC-PC axis, which is multiplied by the serum dilution factor.

Titre factors lower than 0.25 and higher than 3.5 (x-axis) are not taken into consideration in these calculations.

Exemplary quantitative calculation:

Negative Control NC	Extinction
1.Value	0.041
2.Value	0.056
Mean	**0.049**

Positive Control PC	Extinction
1.Value	1.220
2.Value	1.176
Mean	**1.198**



Sample	Dilution of Sample	Extinction (average)	Titre factor (see graphic)	Titre	Interpretation
Serum B	1:1000	2.82	> 3.5	-	positive
	1:10000	1.45	3.05	1:(10000 × 3.05) => 1:30 500	positive
	1:100000	0.210	0.3	1:(100000 × 0.3) => 1:30000	positive

If an electronic analysis program is used, NC and PC have to be set as standards with the titer values of 0 and 2500. The program calculates the titer of the unknown sample. The dilution factor of the sample has to be taken into account.

Interpretation Of Results

This ELISA Kit is used as a complementary test to a classical microbiological detection of infection with Pseudomonas aeruginosa. If a sample is tested positive for antibodies against one of the three Pseudomonas aeruginosa exotoxins, this is a sign of corresponding infection. The evaluation of the measured antibody titres has to be done according to the scheme below.

< 1:500.....Negative

1:500 to 1: 1250....Borderline

> 1:1250.....Positive

Precision

Intra-Assay Variance

Serum samples were diluted 1:1000 and measured 16-fold within one assay. Mean variance was 4.95, 4.49 and 8.41% for alkaline Protease, Elastase and Exotoxin A, respectively.

	Sample 1			Sample 2			Sample 3		
Determinations [n]	16	16	16	16	14	16	16	16	16
Mean Titre	806	529	715	3858	5521	5673	1453	1530	1381
SD	35	43	91	173	135	127	88	44	142
CV [%]	4.3	8.14	12.71	4.47	2.45	2.25	6.08	2.86	10.29

Inter-Assay Variance

Samples were portioned and stored at -20°C for up to 12 years and measured irregularly during this time. The variability measured as coefficient of variation does not exceed 20% for any antigen or sample.

	Sample 1			Sample 2			Sample 3		
Determinations [n]	73	73	72	295	295	293	112	112	112
Mean Titre	1041	958	804	1743	1519	1239	2971	4071	4343
SD	67	60	70	228	231	176	164	309	392
CV [%]	6.39	6.31	8.69	13.09	15.22	14.22	5.51	7.59	9.03

Sensitivity

The analytical sensitivity as a measure for the minimal amount of specific antibody detectable by this test system was determined by the signal variability of the negative control. Therefore, the recalculated antibody titre of the negative control was evaluated and the 3-fold standard deviation was used to determine the limit of detection. The results are shown in table below. The limit of detection of antibody titers for AP, ELA, Exo A in 1:1000 diluted samples is < 1:300.

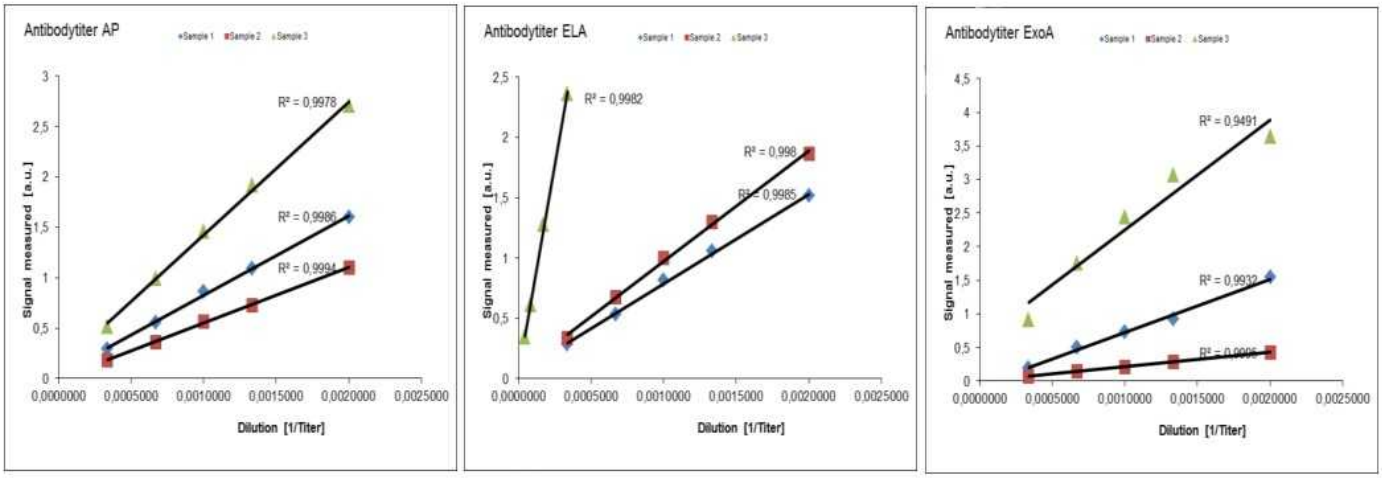
Dilution	AP	ELA	Exo A
-	0.105	0.268	0.181
1:100	10	27	18
1:1000	105	268	181

Specificity

The analytical specificity was assessed by the evaluation of the microbiological status of serologically and microbiologically Pseudomonas-negative cystic fibrosis patients. Most frequently infections with *Staphylococcus aureus, Candida albicans* and *Hemophilus influenza* were detected. These infections do not result in antibodies cross reacting with *Pseudomonas aeruginosa* antigens.

Linearity

Linearity of sample dilution was evaluated by dilution dependent signal decrease in three serum samples with high, medium and low antibody titres. In figure below the measured signals are shown in dependence of the sample dilution. Linear regression analysis revealed coefficients of correlation of >0.9 for all tested samples. The recalculated antibody titres show a good linearity, too.



Interferences

Interference was not investigated, no information regarding the influence of triglycerides, hemoglobin or bilirubin is available. Therefore lipaemic, hemolytic or icteric samples should not be used for this assay.

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.
6. Following components contain human serum: PC1, PC2, PC3, NC, CTR

   Source human serum for the Controls provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

Limitations

Basically, the result of immunological test systems can be affected by various sample components such as

medications or lipids. Their influence is reduced by the assay design, but cannot be excluded completely. In acute and chronic P. aeruginosa infected patients suffering from immunosuppression, assessment of an infection via the detection of antibodies may be inadequate.