7 TECHNICAL NOTES

Storage Conditions
Store the kit at **2-8°C** until its expiry date.

Storage Life
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.

Preparation of reagents
Bring all reagents to room temperature **(20°C - 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.

Dilution
Use the **Dilution Buffer VP** for the **1:100** dilution of **Conjugate Concentrate KK.** Please dilute only according to daily requirements.

The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest. The **1:20** diluted Washing Buffer **WP** is stable **4 weeks at 2-8°C**. Please dilute only according to daily requirements.

Assay Procedure
When performing the assay, Controls **KS, PK, NK** and the samples should be pipetted as fast as possible (e.g., 15 minutes). To avoid distortions due to differences in incubation times the diluted Conjugate Concentrate **KK** and the **Substrate Solution S** should be added to the plate in the same order and in the same time interval as the samples. **Stopping Solution SL** should be added to the plate in the same order as the Substrate Solution **S.**

Incubation
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 Solution **S**, stabilised Tetramethylbencidine, is photosensitive: storage and incubation in the dark.

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

Washing
Proper washing is of 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 **WP** diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

When using an automatic microtiter plate washer, the respective instructions for 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 is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically 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.

8   ASSAY PROCEDURE

Preparation of Reagents		Dilution
KK	Conjugate Concentrate	**1:100** in Dilution Buffer **VP**
WP	Washingsbuffer	**1:20** with **Aqua dest.** (e.g. 100 mL WP with A.dest. add 2000 mL)

Dilute samples **1:1000** with Dilution Buffer **VP** (qualitative test). For quantitative antibody assays it is recommended to perform serial dilutions of **1:1000**, **1:10000** and **1:100000** with Dilution Buffer **VP**.
Before the assay procedure bring all reagents **to room temperature (20°C -25°C).**
Assay procedure in double determination

Pipette	Reagents	Plate 1 AP	Plate 2 ELA	Plate 3 Exo A
100 µL	Negative Control **NK**	A1/A2	A1/A2	A1/A2
100 µL	Positive Control **PK1**	B1/B2	--	--
100 µL	Positive Control **PK2**	--	B1/B2	--
100 µL	Positive Control **PK3**	--	--	B1/B2
100 µL	Control Serum **KS**	C1/C2	C1/C2	C1/C2
100 µL	Sample Dilution	Pipette in the rest of the wells according to requirements.		
Seal the wells of the 3 microtiterplates with the seeling tape				
Incubation: **2 h** at **37°C**				
3x 300 µL	Aspirate the contents of the wells and **wash 3x** with **300 µL** each Wash Buffer **WP/ well**			in each well
100 µL	**1:100** diluted **Conjugate Concentrate KK**			in each well
Seal the wells of the 3 microtiterplates with the seeling tape				
Incubation: **2 h** at **37°C**				
3x 300 µL	Aspirate the contents of the wells and **wash 3x** with **300 µL** each Wash Buffer **WP/ well**			in each well
100 µL	Substrate Solution **S**			in each well
Incubation: **30 min** in the dark **at RT (20°C -25°C).**				
100 µL	Stopping Solution **SL**			in each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.				

9   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 **NK** should be **below 0.25**. The difference between the extinctions of Negative Controls **NK** and the respective Positive Control **PK** must be **at least 0.6**.

10   EVALUATION OF RESULTS

10.1   Calculation of results

Calculations should be performed for each antigen (each plate) separately! Calculate the average of all multiple values.

10.2   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 PK.

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 $\geq$ 20% but < 50% of positive control, can not be clearly classified and must therefore be assessed as **borderline.**

1: 1000 diluted samples, which extinctions are $\geq$ 50% of PK extinction, are to be classified as **positive.**

Examplary qualitative calculation:

Negative Control NK	Extinction
1.Value	0,041
2.Value	0,056
Mean	**0,049**

Positive Control PK	Extinction
1.Value	1,120
2.Value	1,136
Mean	**1,128**

Calculation

PK – NK	: 1.128 – 0.049	= 1.079
Cut-off (20% of PK-NK)	: 0.2 × 1.079	= 0.216
Borderline (50% of PK-NK)	: 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.

10.3   Quantitative calculation

The evaluation is carried out graphically or via an appropriate evaluation program.

The extinction values of the negative NK and positive PK controls are plotted on the y-axis in a double-linear coordinate system against a titer factor. NK = 0 and PK = 2.5 on the x-axis. For the quantification of the sera values, a straight line is drawn through the NK and PK 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 NK-PK 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.

Examplary quantitative calculation:

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

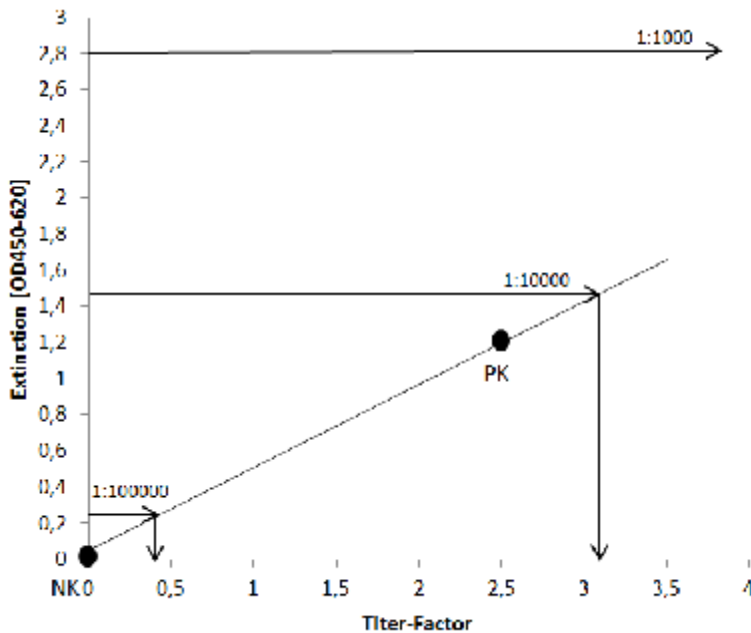| Positive Control PK	Extinction
1.Value	1.220
2.Value	1.176
Mean	**1,198**

Figure 1: Examplary graphic for the quantitative evaluation for serum B.

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

If an electronic analysis program is used, NK and PK 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.

10.4 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. In the literature there are several studies describing the diagnostic performance of the assay (see table 5). These studies often do not differentiate between borderline and positive and apply only one cut-of value (1:500) to differentiate between negative and positive.

Titer	Interpretation
< 1:500	negative
1:500 to 1: 1250	borderline
> 1:1250	positive

For evaluation of the test results, the diagnostic sensitivity and specificity of the test system must be taken into account (see Section 12.7).

The test results should not be the only base for therapeutic decisions. The results should be interpreted in regard to anamnesis, further clinical observations and results of other

diagnostic investigations. It is recommended to consider the international and national guidelines for diagnosisis and treatment.

11 LIMITATION OF PROCEDURE

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. The evaluation of the results must be made in the context of diagnostic sensitivity and specificity of the assay (table 5). In acute and chronic P. aeruginosa infected patients suffering from immunosuppression, assessment of an infection via the detection of antibodies may be inadequate.

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical 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 1.

The limit of detection of antibody titers for AP, ELA, Exo A in 1:1000 diluted samples is < 1:300.

Table 1 Analytical sensitivity / Limit of Detection based on 3-fold standard deviation of the blank. Shown is the recalculated antibody titre of three independent experiments.

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

12.2 Analytical 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.

12.3 Precision

Intra-Assay Variance

Serum samples were diluted 1:1000 and measured 16-fold within one assay. The results are shown in table 2. Mean variance was 4.95, 4.49 and 8.41% for alkaline Protease, Elastase and Exotoxin A, respectively.

Table 2 Intra-Assay Variance

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

Inter-Assay Variance

Samples were portioned and stored at -20°C for up to 12 years and measured irregularly during this time. Table 3 shows the summarized results for 3 different samples. The variability measured as coefficient of variation does not exceed 20% for any antigen or sample.

Table 3 Inter-Assay Variance

	Sample 1			Sample 2			Sample 3		
	AP	ELA	Exo A	AP	ELA	Exo A	AP	ELA	Exo A
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
Number [n]	73	73	72	295	295	293	112	112	112

12.4 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 2 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.
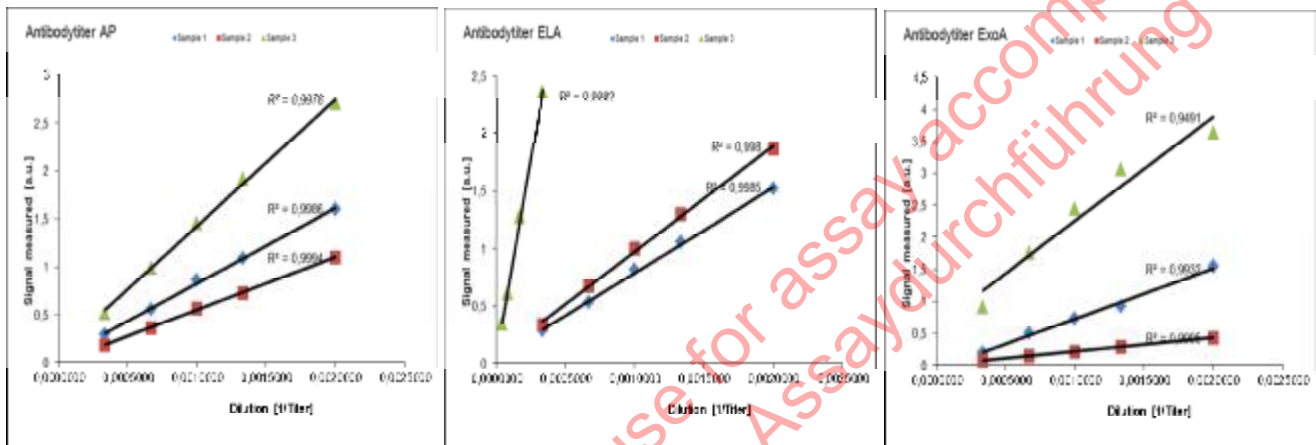
Figure 2 Linearity of sample dilution. Samples with high, medium and low antibody titre were diluted (1:500 – 1:24000) depending on the expected signal intensity and measured results were analysed by linear regression analysis.

12.5 Interference

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.

12.6 Traceability / Assay Calibration

Neither an international standard preparation, purified human Anti-Pseudomonas aeruginosa antibodies nor an established reference method for measurement of these antibodies are available. Thus, the assay calibration is based on manufacturer positive controls with arbitrary antibody titres of 2500 (Mediagnost [1/Titre]). Selected human serum samples with defined antibody content are used for assay production and quality control. This panel allows the traceability of measurement results.

12.7 Diagnostic Quality

For evaluation of diagnostic sensitivity (true positive) and specificity (true negative) a comparison of E15 titre results in serum samples and the microbiological detection of Pseudomonas aeruginosa growth in throat swab samples by classical culture techniques has been conducted.

For the evaluation of the test results regarding Pseudomonas aeruginosa infection only a distinction between positive and negative was made.

Positive

At least one microbiological detection of Pseudomonas aeruginosa within 12 months before the serum sample was collected.

Negative

No microbiological detection of Pseudomonas aeruginosa within 12 months before the serum sample was collected

Table 4 Diagnostic Specificity and Sensitivity Here the absolute numbers of positive and negative results are shown in relation to the microbiological gold standard for different cut-off values (AP: alkaline Protease, ELA: Elastase; Exo A: Exotoxin A; Total: all three antigens). Based on the absolute numbers sensitivity (% true positive) and specificity (% true negative) are calculated).

Cut- off	1:100				1:500				1:1250			
	AP	ELA	Exo A	Total	AP	ELA	Exo A	Total	AP	ELA	Exo A	Total
True Positive **TP**	39	40	43	45	27	31	27	40	19	23	19	32
False Positive **FP**	10	8	11	12	2	2	3	5	1	0	0	1
True Negative **TN**	16	18	15	14	24	24	23	21	25	26	26	25
False Negative **FN**	12	11	8	6	24	20	24	11	32	28	32	19
Sensitivity [%] TP/(TP+FN)	76	78	84	88	53	61	53	78	37	45	37	63
Specificity [%] TN/(TN+FP)	62	69	58	54	92	92	88	81	96	100	100	96

A population of 77 cystic fibrosis patients was investigated, 51 patients were microbiologically positive for Pseudomonas aeruginosa before the serum sample was collected and 26 negative. These results conform to the data published by Kappler et al in 2006. Here the authors used a cut-off Titre of 500 and calculated an overall sensitivity of 86% and a specificity of 96%. In detail it was shown that sensitivity was 64; 53 and 73% for AP, ELA and Exo A, respectively. Specificity for AP, ELA and Exo A was calculated as 99; 100 and 96%. In comparison to our results Kappler et al. detected higher sensitivity and specificity values which probably are the consequence of an increased sample number (183 vs 77).

The Mediagnost test system has been evaluated by different independent research groups regarding its diagnostic sensitivity and specificity. In table 5 the results are summarized. Mean diagnostic specificity is 76% (48 - 96) and mean diagnostic sensitivity is 84% (66–96).

Table 5 Summary of literature results describing diagnostic sensitivity and specificity in dependence of the applied cut-off titre value.

Autor	Journal	Jahr	Mediagnost Assay	Patient [n]	Cut-off Titre	Dignostische Sensitivity [%]	Dignostische Specificity [%]
Kappler et al.	Thorax	2006	AP/ ELA/ Exo A	183	500	86	96
Tramper-Stranders et al.	Thorax	2006	AP/ ELA/ Exo A	220	1250	66	96
					500	79	89
				220	AP > 0 ELA > 35 Exo A > 0	96	79
Ratjen et al.	Pediatric Pulmonol	2007	AP/ ELA/ Exo A	375	AP > 285 ELA > 300 Exo A > 1000	92.7	93.3
Dogru et al.	Turkish Journal of Pediatrics	2013	AP/ ELA/ Exo A	90	500	87.5	70
Anstead et al.	J Cystic Fibrosis	2013	AP/ ELA/ Exo A	304	100	78	48
Daines et al.	J Cystic Fibrosis	2014	AP/ ELA/ Exo A	68	100	66	56

13 LITERATUR / LITERATURE

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14   INTERNATIONALE ASSAY DESCRIPTION

KK	CONJ	1:100 DILU BUF VP		
WP	WASHBUF 20x	1:20 DILU A. dest.		
-	SPE 1:1000 DILU BUF VP ↔			
-	°C 20°C - 25°C ⊕ max. 1 h			
		MTP AP	MTP ELA	MTP Exo A
100 µL	Control NEG NK	A1/A2	A1/A2	A1/A2
100 µL	Control POS PK1	B1/B2	--	--
100 µL	Control POS PK2	--	B1/B2	--
100 µL	Control POS PK3	--	--	B1/B2
100 µL	CONTROL KS	C1/C2	C1/C2	C1/C2
100 µL	SPE 1:1000 DILU BUF VP			
	TAPE			
	⊕ 2 h °C 37°C			
3x 300 µL	3x WASHBUF WP			
100 µL	CONJ KK 1:100 DILU VP			
	TAPE			
	⊕ 2 h °C 37°C			
3x 300 µL	3x WASHBUF WP			
100 µL	SUBST TMB S			
	⊕ 0.5 h °C 20°C – 25 °C ☀			
	H₂SO₄ SL			
	MEASURE			

15   ASSAY PROCEDURE

Preparation of Reagents		Dilution
KK	**Conjugate Concentrate**	**1:100** in Dilution Buffer **VP**
WP	**Washingsbuffer**	**1:20** with **Aqua dest.** (**e.g. 100 mL WP** with **A.dest.** add **2000 mL**)

Dilute samples **1:1000** with Dilution Buffer **VP** (qualitative test). For quantitative antibody assays it is recommended to perform serial dilutions of **1:1000**, **1:10000** and **1:100000** with Dilution Buffer **VP**.

Before the assay procedure bring all reagents **to room temperature (20°C -25°C).**

Assay procedure in double determination

Pipette	Reagents	Plate 1 AP	Plate 2 ELA	Plate 3 Exo A
100 µL	Negative Control **NK**	A1/A2	A1/A2	A1/A2
100 µL	Positive Control **PK1**	B1/B2	--	--
100 µL	Positive Control **PK2**	--	B1/B2	--
100 µL	Positive Control **PK3**	--	--	B1/B2
100 µL	Control Serum **KS**	C1/C2	C1/C2	C1/C2
100 µL	Sample Dilution	Pipette in the rest of the wells according to requirements.		
Seal the wells of the 3 microtiterplates with the seeling tape				
Incubation: **2 h** at **37°C**				
3x 300 µL	Aspirate the contents of the wells and **wash 3x** with **300 µL** each Wash Buffer **WP/ well**			in each well
100 µL	**1:100** diluted **Conjugate Concentrate KK**			in each well
Seal the wells of the 3 microtiterplates with the seeling tape				
Incubation: **2 h** at **37°C**				
3x 300 µL	Aspirate the contents of the wells and **wash 3x** with **300 µL** each Wash Buffer **WP/ well**			in each well
100 µL	Substrate Solution **S**			in each well
Incubation: **30 min in the dark at RT (20°C -25°C).**				
100 µL	Stopping Solution **SL**			in each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.				

This assay procedure is a duplicate of the page 24.