Human PMN Elastase Platinum ELISA

ALX-850-265

Enzyme-linked Immunosorbent Assay for quantitative detection of human PMN Elastase.

For research use only.

Not for diagnostic or therapeutic procedures.

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TRADEMARKS AND PATENTS

Several of Enzo's products and product applications are covered by US and foreign patents and patents pending.

TABLE OF CONTENTS

1. Intended Use

The human PMN elastase ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human PMN elastase. **The human PMN elastase ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2. Summary

The human organism reacts with an inflammatory response to attacks of invading pathogens (microorganisms and viruses) or damaged tissue (after accidents or surgery). Polymorphonuclear (PMN) granulocytes play an important role as primary defence cells in this inflammatory reaction. Different bloodstream mediators (cytokines, leukotrienes, complement factors, bacterial endotoxins, clotting and fibrinolysis factors) attract and stimulate these cells to phagocytize and destroy not naturally occurring agents.

PMN granulocytes use proteinases to digest these agents and tissue debris. One of these proteinases is PMN elastase which is localised in the azurophilic granules of the polymorphonuclear granulocytes. During phagocytosis of foreign substances these enzymes are also partially excreted into the extracellular surrounding, where the activity of PMN elastase is regulated by inhibitors (esp. the α_1-proteinase inhibitor, α_1-PI). An overwhelming release of PMN elastase, however, can exceed the inhibitory potential of the α_1-proteinase inhibitor. Thus, enzymatically active PMN elastase, together with simultaneously produced oxidants (O_2-radicals, H_2O_2, OH-radicals), can cause local tissue injury.

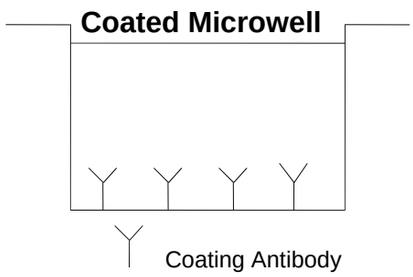
Due to the bloodstream and lymphatic system, however, α_1-PI is delivered subsequently and eventually able to form a complex with all excreted elastase. Therefore, the concentration of the PMN elastase/α_1-PI complex correlates with the released PMN elastase and can be used as a measure for the activity of granulocytes during an inflammatory response.

Primarily, determinations of PMN elastase find its application in observation of the course of trauma, shock and sepsis. Further indications are the areas of hemodialysis, infections by obstetrics, joint diseases, effusions of sport injuries, intestinal affection, pancreatitis, cystic fibrosis and male adnex affections.

3. Principles of the Test

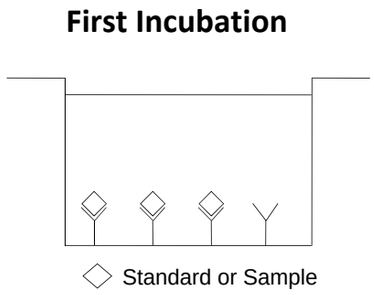
An anti-human PMN Elastase coating antibody is adsorbed onto microwells.

Figure 1



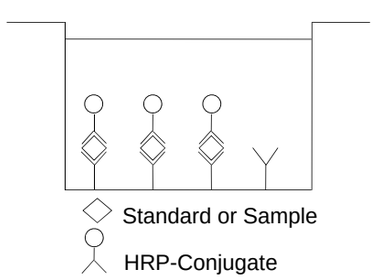
Human PMN Elastase present in the sample or standard binds to antibodies adsorbed to the microwells.

Figure 2



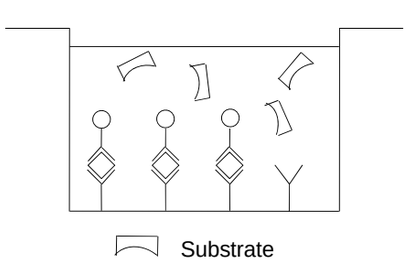
Following incubation unbound biological components are removed during a wash step and a HRP-conjugated anti-α_1-PI antibody is added and binds to human PMN elastase/α_1-PI complex captured by the first antibody.

Figure 3



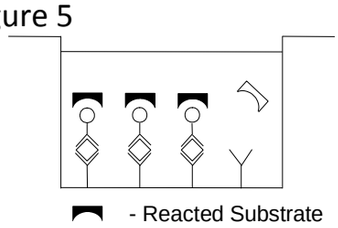
Following incubation unbound HRP-conjugated anti-α_1-PI antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 4



A colored product is formed in proportion to the amount of human PMN Elastase present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human PMN Elastase standard dilutions and human PMN Elastase concentration determined.

Figure 5



4. Reagents Provided

1 aluminium pouch with a **Microwell Plate coated** with polyclonal antibody to human PMN Elastase

1 vial (16 ml) **HRP-Conjugate** anti-α_1-PI polyclonal antibody, ready to use

1 vial human PMN Elastase **Standard** lyophilized, 10 ng/ml upon reconstitution

1 vial **Control high,** lyophilized

1 vial **Control low,** lyophilized

1 bottle (50 ml) **Sample Diluent**

1 bottle (50 ml) **Wash Buffer Concentrate** (10x)

1 bottle (22 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (7 ml) **Stop Solution** (1M hydrochloric acid)

2 **Adhesive Films**

5. Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C except controls. Store lyophilized controls at -20°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C), or to -20°C, respectively. Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6. Specimen Collection and Storage Instructions

Cell culture supernatants, plasma, exudate, bronchoalveolar lavage fluid, cerebrospinal fluid and seminal plasma were tested with this assay. Other body fluids might be suitable for use in the assay. Separate plasma from cells by centrifugation.

Pay attention to a possible **“Hook Effect”** due to high sample concentrations (see chapter 11).

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human PMN Elastase. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 0).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7. Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8. Precautions for Use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. Preparation of Reagents

Buffer Concentrate should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrate**, warm it gently until they have completely dissolved.

9.1. Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (10x) into a clean 1000 ml graduated cylinder. Bring to final volume of 500 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (10x) (ml)	Distilled Water (ml)
1 - 6	25	225
1 - 12	50	450

9.2. Human PMN Elastase Standard

Reconstitute **human PMN Elastase standard** by addition of Sample Diluent 30 min. before use. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted standard = 10 ng/ml).

Aliquots can be stored at –20°C.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.2.1).

9.2.1. External Standard Dilution

Label 6 tubes, one for each standard point.

S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

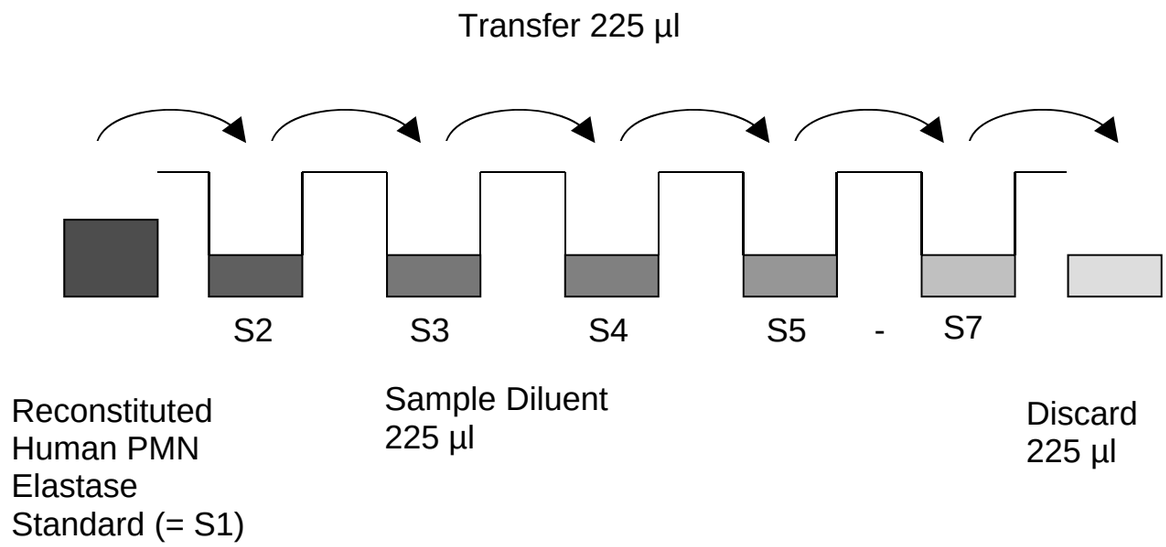
Pipette 225 µl of Sample Diluent into tubes S2 – S7.

Pipette 225 µl of reconstituted (serves as the highest standard S1, concentration of standard 1= 10 ng/ml) into the first tube, labeled S2, and mix (concentration of standard 2 = 5 ng/ml).
Pipette 225 µl of this dilution into the second tube, labeled S3, and mix thoroughly before the next transfer.

Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 6).

Sample Diluent serves as blank.

Figure 6



9.3. Controls

Reconstitute by adding 1 ml Sample Diluent to lyophilized **controls** 30 minutes before use. Further treat the controls like your samples in the assay. For control range please refer to certificate of analysis or vial label. Store reconstituted controls aliquoted at -20°C. Avoid repeated freeze and thaw cycles.

10. Test Protocol

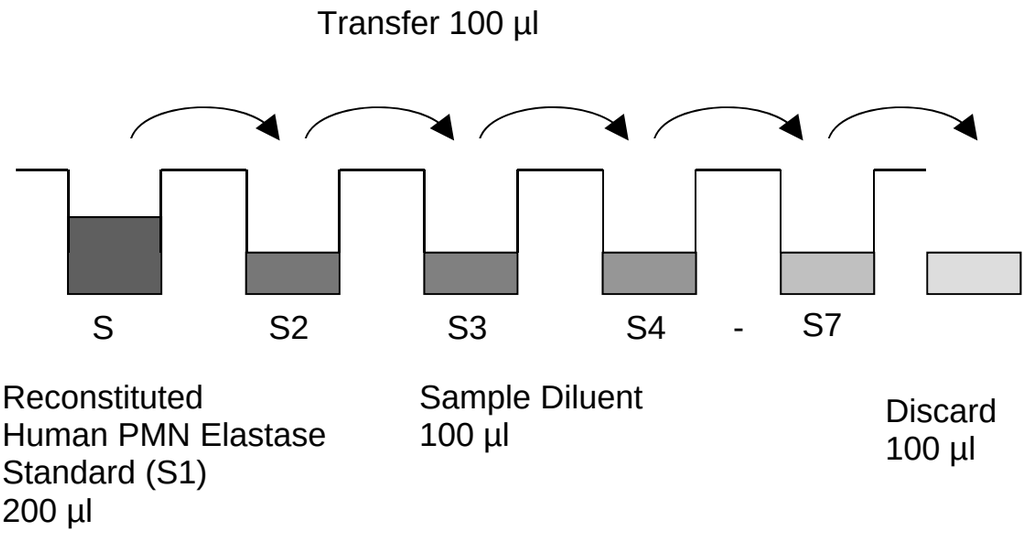
a. Predilute your samples before starting with the test procedure. Dilute samples 1:100 with Sample Diluent according to the following scheme:
   Dilution 1: 10 µl sample + 90 µl Sample Diluent
   Dilution 2: 50 µl of dilution 1 + 450 µl Sample Diluent

b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.2.1):
   Add 100 µl of Sample Diluent in duplicate to to **standard wells** B1/2- G1/2, leaving A1/A2 empty. Pipette 200 µl of prepared **standard** (see Preparation of Standard 9.2, concentration of S1 = 10.00 ng/ml) in duplicate into well A1 and A2 (see Table 1).

Transfer 100 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection (concentration of standard, S2 = 5.00 ng/ml), and transfer 100 µl to wells C1 and C2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human PMN Elastase standard dilutions ranging from 10.00 to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7



In case of an **external standard dilution** (see 9.2.1), pipette 100 µl of these standard dilutions (S1 – S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Sample 3	Sample 3
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.31 ng/ml)	Standard 6 (0.31 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

d. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.

e. Add 100 µl of each prediluted **sample** in duplicate to the **sample wells**.

f. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.

g. Remove adhesive film and empty wells. Wash the microwell strips 4 times with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry.**

h. Add 150 µl of **HRP-Conjugate,** ready to use to all wells.

i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 200 rpm.

j. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point g. of the test protocol. Proceed immediately to the next step.

k. Pipette 200 µl of **TMB Substrate Solution** to all wells.

l. Incubate the microwell strips at room temperature (18° to 25°C) for about 20 min. Avoid direct exposure to intense light.

   The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable.

   Determination of the ideal time period for color development has to be done individually for each assay.

   It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

m. Stop the enzyme reaction by quickly pipetting 50 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

n. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11. Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human PMN Elastase concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human PMN Elastase for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human PMN Elastase concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:100, the concentration read from the standard curve must be multiplied by the dilution factor (x 100).**
- **Calculation of 1:100 prediluted samples with a concentration exceeding standard 1 may result in incorrect, low human PMN Elastase levels (Hook Effect). Such samples require further external predilution according to expected human PMN Elastase values with Sample Diluent in order to precisely quantitate the actual human PMN Elastase level.**
- It is suggested that each testing facility establishes a control sample of known human PMN Elastase concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for human PMN Elastase ELISA. Human PMN Elastase was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

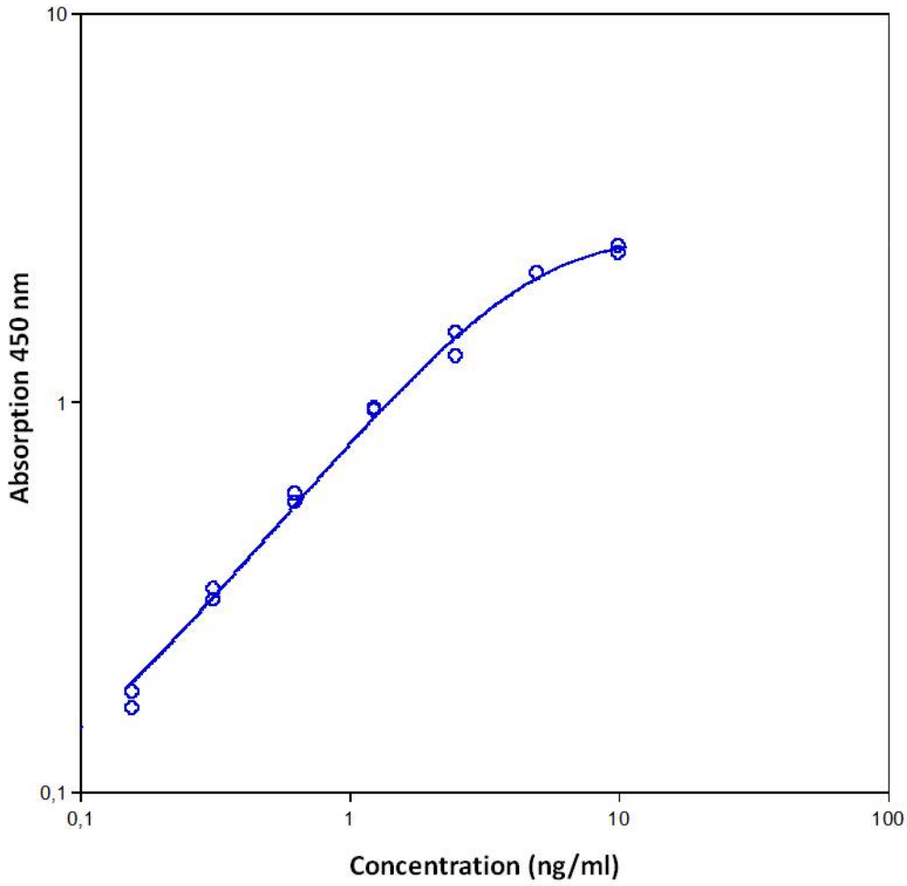


Table 2

Typical data using the human PMN Elastase ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human PMN Elastase Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	10.00	2.452	2.516	2.5
		2.579		
2	5.00	2.184	2.192	0.3
		2.199		
3	2.50	1.548	1.451	6.7
		1.354		
4	1.25	1.005	1.003	0.2
		1.001		
5	0.63	0.629	0.613	2.6
		0.598		
6	0.31	0.377	0.366	2.8
		0.356		
7	0.16	0.226	0.218	3.6
		0.210		
Blank	0	0.048	0.047	3.3
		0.046		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

12. Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13. Performance Characteristics

13.1. Sensitivity

The limit of detection of human PMN Elastase defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 1.98 ng/ml (mean of 6 independent assays).

13.2. Reproducibility

13.2.1. Intra-assay

Reproducibility within the assay was evaluated in 10 independent experiments. Each assay was carried out with 10 replicates of 3 plasma samples containing different concentrations of human PMN Elastase. Two standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 4.8%.

13.2.2. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 10 independent experiments. Each assay was carried out with 10 replicates of 4 plasma samples containing different concentrations of human PMN Elastase. Two standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 5.6%.

13.3. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human PMN elastase into a plasma sample. Recoveries were determined in 3 independent experiments with 4 replicates each.

The unspiked plasma was used as blank in these experiments.

The recovery ranged from 96% to 110% with an overall mean recovery of 104% (see Table 3).

Table 3

Sample	Spiking Solution	Expected PMN elastase Concentration (ng/ml)	Observed PMN elastase Concentration (ng/ml)	Recovery of Expected PMN elastase Concentration (%)
1	-	-	23.2	-
	A	69.4	72.4	104
	B	54.1	59.3	109
	C	47.2	49.6	101
2	-	-	30.6	-
	A	76.7	73.4	96
	B	61.4	59.3	97
	C	54.4	56.8	104
3	-	-	61.7	-
	A	107.8	118.0	109
	B	92.5	100.8	109
	C	85.6	94.8	110

13.4. Dilution Parallelism

Plasma samples with different levels of human PMN elastase were analysed at serial 2 fold dilutions with 4 replicates each.

The recovery ranged from 87% to 114% with an overall recovery of 96.5% (see Table 4).

Table 4

Sample	Dilution	Expected PMN elastase Concentration (ng/ml)	Observed PMN elastase Concentration (ng/ml)	Recovery of Expected PMN elastase Concentration (%)
1	1:100	-	114.0	-
	1:200	55.7	57.9	103
	1:400	27.8	31.8	114
	1:800	13.9	14.6	105
2	1:100	-	135.6	-
	1:200	67.8	66.5	98
	1:400	33.9	30.8	91
	1:800	16.9	18.8	111
3	1:100	-	255.0	-
	1:200	127.5	130.5	102
	1:400	63.8	61.0	96
	1:800	31.9	31.9	100
4	1:100	-	540.1	-
	1:200	270.0	246.2	92
	1:400	135.0	119.9	89
	1:800	67.5	61.7	91
5	1:100	-	641.8	-
	1:200	320.9	281.4	88
	1:400	160.4	149.7	93
	1:800	80.2	69.9	87
6	1:100	-	909.5	-
	1:200	454.7	444.5	98
	1:400	227.4	208.1	92
	1:800	113.7	100.4	88

13.5. Specificity

The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human PMN Elastase positive sample.

There was no cross reactivity or interference detected.

13.6. Expected Values

A panel of 57 plasma samples from randomly selected apparently healthy donors (males and females) was tested for human PMN elastase. The detected human PMN elastase mean level was 35 ng/ml. The levels measured may vary with the sample collection used.

14. Reagent Preparation Summary

14.1. Wash Buffer (1x)

Add **Wash Buffer Concentrate** 10x (50 ml) to 450 ml distilled water.

Number of Strips	Wash Buffer Concentrate (10x) (ml)	Distilled Water (ml)
1 - 6	25	225
1 - 12	50	450

14.2. Human PMN Elastase Standard

Reconstitute lyophilized **human PMN Elastase standard** with Sample Diluent 30 minutes before use. (Reconstitution volume is stated on the label of the standard vial.)

14.3. Controls

Add 1 ml Sample Diluent to lyophilized **controls**.

15. Test Protocol Summary

1. Predilute sample with Sample Diluent 1:100.
2. Determine the number of microwell strips required.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells leaving the first wells empty. Pipette 200 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.2.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 100 µl prediluted sample in duplicate, to designated sample wells.
6. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
7. Empty and wash microwell strips 4 times with Wash Buffer.
8. Add 150 µl HRP-Conjugate to all wells.
9. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
10. Empty and wash microwell strips 4 times with Wash Buffer.
11. Add 200 µl of TMB Substrate Solution to all wells.
12. Incubate the microwell strips for about 20 minutes at room temperature (18° to 25°C).
13. Add 50 µl Stop Solution to all wells.
14. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:100, the concentration read from the standard curve must be multiplied by the dilution factor (x 100).

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