Technical Hints

- It is recommended that all standards and samples be assayed in duplicate.
- It is important that Wash Buffer B reflects the environment of the samples being measured. To abrogate the matrix effect of sputum sol (when produced as described above), a minimum **required dilution** of x50 is recommended. Other suitable dilutions include x100, x200 and x400. Dilutions above x400 need further investigation to determine suitability in this assay
- Following dilution, samples must be kept on ice until pipetted onto the immunoassay plate
- To avoid cross-contamination, change pipette tips between addition of each standard, sample and reagents. Also, use separate reservoirs for each reagent
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary
- We recommend the use of external controls for internal quality control.

Precautions

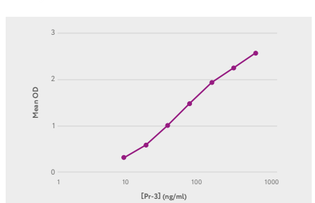
- Wear personal protective equipment including gloves, clothing, eye and face protection. Wash hands after handling reagents
- Pr-3Tag is supplied in dimethylformamide (DMF) which is a solvent classified as harmful and an irritant. Wear gloves and protective clothing when handling and avoid breathing fumes
- Stop Solution is an acidic solution
- The wash buffers and diluents within the kit contain preservatives which may cause an allergic skin reaction. Do not breathe mist
- TMB substrate is classified as an irritant and can cause respiratory tract, eye and skin irritation.

Performance Characteristics

Typical Results:

This standard curve is provided for illustrative purposes only.

A standard curve should be generated for each set of samples analysed.



Measuring Range:

The range of the standard curve of this immunoassay is 7.81 – 500 ng/ml. It is essential the final measurement falls within the linear range of the standard curve. Sample readings above or below the linear range should be concentrated or diluted.

Limit of Detection:

The lowest concentration of Pr-3 giving an absorbance reading greater than two standard deviations (SD) above the mean zero (blank matrix) reading (n=32) was determined to be 2.35 ng/ml.

Precision:

Intra-assay coefficient of variation (CV) was determined by measurement of 24 replicates of three diluted sputum sol samples (high, medium and low) on one analytical assay run.

Intra-Assay CVs (within assay precision) = 6.9%.

Inter-assay CV was determined by measurement of duplicates of eight diluted sputum sol samples on six analytical assay runs performed by three independent operators over two days.

Inter-Assay CV (between assay precision) = 21.4%.

Analytical Recovery:

To assess the recovery of the assay, active Pr-3 (150 ng/ml) was spiked into five sputum sol samples, before dilution x100 and measurement on the assay. Observed active Pr-3 concentrations were then compared to baseline Pr-3 in each sample.

Average recovery = 97.9%.

Analytical Linearity:

To assess the linearity of the assay, five sputum sol samples, spiked with active Pr-3 (150 ng/ml), were diluted x100, before being serially diluted and measured on the assay.

Dilution Factor	Average % Expected Value
X2	106.4%
X4	110.0%
X8	108.6%
X16	118.0%

Specificity:

To assess cross-reactivity, active neutrophil elastase (0.4 µg/mL), active cathepsin G (0.2 µg/mL) and alpha-1-antitrypsin (10 µg/mL) were spiked separately into wash buffer B and run as an unknown in the assay. No cross-reactivity was observed.

To assess interference, seven diluted sputum sol samples were analysed in the assay with or without spiked active neutrophil elastase (0.4 µg/mL) or active cathepsin G (0.2 µg/mL). Significant interference was not found. The average recovery of active Pr-3 after spiking with active neutrophil elastase and active cathepsin G was 74.1% and 134.9%, respectively.

Processing using Sputolysin® (Dithiothreitol (DTT)) significantly affects the quantification of active Pr-3 on the assay.

Disposal

Sputum/sputum sol samples and used kit components are potentially biohazardous. Dispose appropriately in line with local clinical waste guidelines.

Troubleshooting

For help or advice please contact ProAxsis Ltd on **+44 (0) 2890 730445** or **info@proaxsis.com**

References

1. Korkmaz, B., Moreau, T. and Gauthier, F. (2008) Neutrophil elastase, proteinase 3 and cathepsin G: Physiochemical properties, activity and physiopathological functions. *Biochimie,* 90: 227-242
2. Twigg, M.S., Brockbank, S., Lowry, P., FitzGerald, S.P., Taggart, C. and Weldon, S. (2015) The role of serine proteases and antiproteases in the cystic fibrosis lung. *Mediators of Inflammation*, 293053
3. Sinden, N.J. and Stockley, R.A. (2013) Proteinase 3 activity in sputum from subjects with alpha-1-antitrypsin deficiency and COPD, *European Respiratory Journal,* 41: 1042-1050
4. Witko-Sarsat, V., Halbwachs-Mecarelli, L., Schuster, A., Nusbaum, P., Ueki, I., Cateloup, S., Lenoir, G., Descamps-Latscha, B. and Nadel, J. (1999) Proteinase 3, a potent secretagogue in airways, is present in cystic fibrosis sputum. *American Journal of Respiratory Cell and Molecular Biology,* 20: 729-736.

Symbols Used

CE	EC Declaration of Conformity		Manufactured By
RUO	Research Use Only		Expiry Date
REF	Catalogue Number		Storage Upper Limit of Temperature
LOT	Lot Number		Storage Temperature Limitations
	Consult Instructions		

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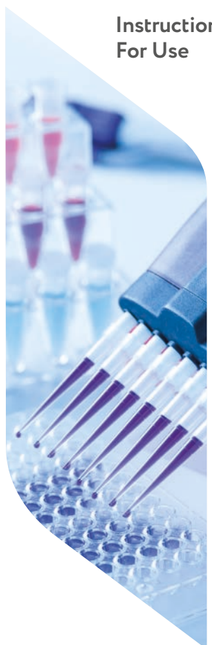
Version 1. December 2020

ProAxsis

ProteaseTag® Active Proteinase 3 Immunoassay

Catalogue Number PA008US

Instructions For Use



Intended Use

For the **in vitro** quantitative determination of active human proteinase 3 in sputum samples.

Read instructions for use in full before using this product.

For professional use only.

CE RUO

BACKGROUND

Proteinase 3 (Pr-3), also known as myeloblastin, is a chymotrypsin-like serine protease primarily derived from primary (azurophil) granules of polymorphonuclear neutrophils (PMNs). Other neutrophil serine proteases include neutrophil elastase (NE) and cathepsin G (CatG) which, alongside Pr-3, have numerous biological functions, including the elimination of pathogens[1].

In pulmonary disease, excessive proteolytic tissue degradation damages the airway walls contributing to a destructive and vicious inflammatory cycle[2]. Active Pr-3 levels in sputum have been shown to correlate with biomarkers of inflammation in respiratory diseases including cystic fibrosis and chronic obstructive pulmonary disease[3,4]. Measurement of this biomarker in sputum may aid in the understanding of these pulmonary diseases.

Assay Principle

The ProteaseTag® Active Proteinase 3 Immunoassay utilizes ProteaseTag® technology to specifically detect and quantify active Pr-3.

During a short incubation, the Pr-3Tag is coated onto the immunoassay plate before washing to remove excess. Standards and samples are added, and the Pr-3Tag will interact with the active site of Pr-3 during a second incubation. Active Pr-3 present in the solution will irreversibly bind to the Pr-3Tag while latent and endogenous inhibitor bound Pr-3 will be removed by washing. A primary anti-Pr-3 antibody is added to the wells and incubated. This antibody binds to a site on the active Pr-3 that is distal to the active site. Unbound antibody is removed by washing. A secondary horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody is added to each test well and incubated. This detection antibody attaches to the bound primary anti-Pr-3 antibody with unbound secondary antibody subsequently removed by washing. A colour forming substrate containing tetramethylbenzidine (TMB) is added to each test well and reacts with HRP to generate a blue coloured product. This enzymatic reaction is subsequently stopped by the addition of acidic stop solution to each test well (a yellow solution is formed). The colour intensity (absorbance) is read at 450 nm using a plate reader.

Materials Provided

Freezer Box Components	
Pr-3Tag	1 vial containing 30 µl (concentration 1 mM) of capture probe in dimethylformamide (DMF)
Pr-3 Standard	2 vials containing 10 µl of active Pr-3 (concentration 0.05 mg/ml)
Secondary Pr-3 Antibody	1 vial containing 30 µl of anti-mouse IgG conjugated to horse radish peroxidase (HRP) in a stability solution

Main Box Components	
Immunoassay Plate	96 well streptavidin-coated microtitre plate (pre-blocked)
Pr-3 Wash Buffer A Concentrate	32 ml of a 25-fold concentration of buffered surfactant with preservatives
Pr-3 Wash Buffer B Concentrate	24 ml of a 10-fold concentration of buffered surfactant with preservatives
Primary Pr-3 Antibody	1 vial containing 30 µl anti-Pr-3 mouse monoclonal antibody in a stability solution
TMB Substrate	12 ml of tetramethylbenzidine solution (ready to use)
Stop Solution	6 ml of 2 N sulphuric acid (ready to use)
Plate Sealers	5 adhesive strips

Storage

Store the freezer box immediately upon receipt at -20°C.

Store the main box immediately upon receipt at 2-8°C.

Storage of Opened Kits

Concentrated buffers, TMB Substrate, Stop Solution, Pr-3Tag, Primary Pr-3 Antibody and Secondary Pr-3 Antibody may be used twice within seven days of initial opening provided they have been returned to recommended storage conditions immediately after use and that this is within the expiration date of the kit.

Immunoassay plates may be used twice within seven days of initial opening provided the ensuing procedure is followed: Unused wells should be covered by a plate seal during initial assay procedure. After initial analysis, excess fluid should be removed from used wells, before being covered by a plate seal. Seal should be removed from unused wells and the immunoassay plate returned to the foil pouch containing the desiccant pack, resealed along the edge and returned to storage at 2-8°C.

Each vial of Pr-3 Standard can only be used once.

Materials Required but not Supplied

- Deionized or distilled water
- Calibrated pipettes and corresponding pipette tips
- Tubes and tube racks
- Clean containers for diluted wash buffers
- Graduated cylinder
- Vortex mixer
- Incubator (37°C)
- Wash bottle or automated microplate washer
- Calibrated microtitre plate reader capable of reading at 450 nm
- Computer and computer software for data analysis.

Sample Preparation

After collection, it is recommended that sputum is kept on ice (4°C) before processing into cell free sputum supernatant (sputum sol). To produce sputum sol: Dilute sputum x5 with chilled phosphate buffered saline (pH 7.4), mix by inversion (x10), centrifuge at 3000 *g* for 30 minutes at 4°C, before supernatant removal, mixing and aliquoting. It is suggested that sputum sol samples are stored long term at -80°C.

Reagent Preparation

- Pr-3Tag
  - To be prepared prior to Step 5 of the assay procedure
  - Dilute 24 µl of Pr-3Tag with 11976 µl of Wash Buffer A to prepare 12 ml of Pr3-Tag solution (500-fold dilution)
- Pr-3 Standard
  - To be prepared prior to Step 7 of the assay procedure
  - **Must be kept on ice at all times prior to use**
  - Standard curve preparation:
    - Pipette 495 µl of Wash Buffer B into the first micro-tube
    - Pipette 250 µl of Wash Buffer B into 6 subsequent micro-tubes
    - Add 5 µl of the Pr-3 Standard to the first tube. Carry out a dilution series by transferring 250 µl from one tube to the next. Mix each tube thoroughly before transferring to next tube. This will create a calibration curve with the following Pr-3 concentrations: 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 ng/ml
    - Use Pr-3 Wash Buffer B as the zero (blank) standard
- Sputum Sol Samples
  - To be prepared prior to Step 7 of the assay procedure
  - **Must be kept on ice at all times prior to use**
  - Refer to sputum sol preparation described above
  - Dilute by at least the minimum required dilution with Wash Buffer B
- Primary Pr-3 Antibody
  - To be prepared prior to Step 9 of the assay procedure
  - Dilute 24 µl of Primary Pr-3 Antibody with 11976 µl of Wash Buffer A to prepare 12 ml of Anti-Pr-3 Antibody solution (500-fold dilution)
- Secondary Pr-3 Antibody
  - To be prepared prior to Step 11 of the assay procedure
  - Dilute 24 µl of Secondary Pr-3 Antibody with 11976 µl of Wash Buffer A to prepare 12 ml of Secondary Pr-3 Antibody solution (500-fold dilution).

Assay Procedure

Volumes listed below are suitable for full plate analysis. Reduce if appropriate.

1. Dilute 32 ml of Pr-3 Wash Buffer A Concentrate with 768 ml deionised water to prepare 800 ml of Wash Buffer A. Mix thoroughly
2. Dilute 24 ml of Pr-3 Wash Buffer B Concentrate with 216 ml deionised water to prepare 240 ml of Wash Buffer B
3. Remove plate from the foil pouch
4. Aspirate and wash each well using 300 µl/well Wash Buffer A. Repeat the procedure twice for a total of 3 washes
5. Add 100 µl of diluted Pr3-Tag into each well. Cover with the plate sealer and incubate at 37°C for 30 minutes
6. Aspirate and wash each well using 300 µl/well **Wash Buffer B**. Repeat the procedure twice for a total of 3 washes
7. Add 100 µl of prepared standards, samples and blank into each well. Cover with the plate sealer and incubate at 37°C for 60 minutes
8. Aspirate and wash each well using 300 µl/well Wash Buffer A. Repeat the procedure twice for a total of 3 washes
9. Add 100 µl of diluted Primary Pr-3 Antibody into each well. Cover with the plate sealer and incubate at 37°C for 60 minutes
10. Aspirate and wash each well using 300 µl/well Wash Buffer A. Repeat the procedure twice for a total of 3 washes
11. Add 100 µl of diluted Secondary Pr-3 Antibody into each well. Cover with the plate sealer and incubate at 37°C for 60 minutes
12. Aspirate and wash each well using 300 µl/well Wash Buffer A. Repeat the procedure twice for a total of 3 washes
13. Add 100 µl of TMB Substrate solution into each well. Cover with the plate sealer and incubate at 37°C for 10 minutes. **Protect from light**
14. Add 50 µl of Stop Solution to each well. The colour within the wells should change from blue to yellow
15. Determine the optical density of each well within 10 minutes using a microplate reader set to 450nm.

Calculation of Results

Average the duplicate readings for each standard, control and sample and subtract the zero blank from the final results. Construct a standard curve by plotting the mean absorbance on the y-axis against active Pr-3 concentration on the x-axis. Generate a curve using a four-parameter logistic fit. Alternatively, the data may be linearised by plotting the log of the active Pr-3 concentration versus the mean absorbance. If samples have been diluted, the concentration reported should be corrected for the dilution factor.

Limitations of the Assay

- Store all components correctly.
- Use all unopened reagents before their expiry date.
- Do not mix or substitute reagents with those from other lots or sources.
- All procedures should be carried out in accordance with the protocol. Performing the assay outside of the prescribed time and temperature ranges may produce invalid data.
- This immunoassay has been optimized and validated for use with human sputum only. For use with other sample types, the user should confirm sample characteristics and validate kit performance with the sample type they intend to analyze.