Development of the first protease multiplex immunoassay for active neutrophilic serine protease biomarkers

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Introduction

The serine proteases, neutrophil elastase (NE) and cathepsin G (Cat G) are regarded as key players in the pathophysiology of various respiratory disorders[1,2], with their measurement becoming increasingly common within the clinical setting. At present, only monoplex assays are commercially available; and although useful, they can be time consuming and often require large sample volumes.

The aim of this study was, therefore, to develop a novel protease multiplex immunoassay for the quantitative determination of active NE and Cat G in biological solutions.

Materials & Methods

NE and Cat G ProteaseTags® (PRX1138 and PRX1186, respectively) were synthesised using a combination of solid-phase peptide synthesis (SPPS), and solution-phase methodologies.

Monoclonal antibodies, raised against human NE and Cat G, were coupled to two spectrally distinct sets of magnetic microspheres (Luminex Corp.), using an antibody coupling kit (Luminex Corp.). Coupling confirmation was subsequently demonstrated upon a short incubation with varying concentrations of the streptavidin-phycoerythrin (SAPE) reporter molecule.

An initial pre-incubation step, between ProteaseTags® and combined NE/Cat G calibration solutions, was performed in a 96-well plate at room temperature, under agitation. To enable capture of complexed proteases, an equal volume of an antibody-microsphere conjugate cocktail was then added to plate wells, and incubated at 800 rpm.

This was followed by a manual wash step, prior to incubation with the SAPE reporter molecule. A further wash step was then performed, followed by analysis on a MagPix® instrument (Luminex).

A range of experiments were subsequently performed to evaluate the degree of non-specific binding (NSB) between components of the multiplex assay. Firstly, each protease was tested against reagents designed to measure the alternative protease, with a general NSB omission assay assessment then carried out on the final multiplex format.

References

1. Polverino, E. (2017) *Chest*, 152(2): 249-262
2. Guerra, M. (2019) *American Chemical Society* 5: 539-548

Results

Simultaneous measurement of NE and Cat G calibration solutions was readily achievable in under 4 hours (Figs. 1A&B). Moreover, transfer to a multiplex platform was found to amplify sensitivity, for both proteases, relative to existing monoplex assays, thereby raising the possibility of NE & Cat G detection in more dilute sample types.

A series of omission experiments were then performed to assess the level of NSB between assay components. NE was found to display negligible NSB with key Cat G components i.e. PRX1186 (Fig. 2A); with low levels of NSB being observed for Cat G, when tested against NE components i.e. PRX1138 (Fig. 2B).

Next, other potential sources of NSB were then evaluated. Figs 3A&B demonstrate that the assay signal, for both analytes, is abrogated upon exclusion of key binding solutions i.e. ProteaseTags®, antibody-microsphere conjugates, protease calibrants and the SAPE reporter molecule.

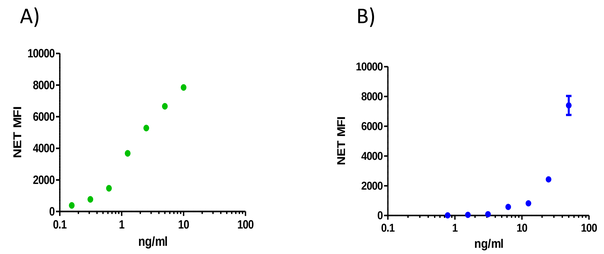

Figure 1. Representative standard curves obtained with A) NE and B) Cat G during multiplex analysis

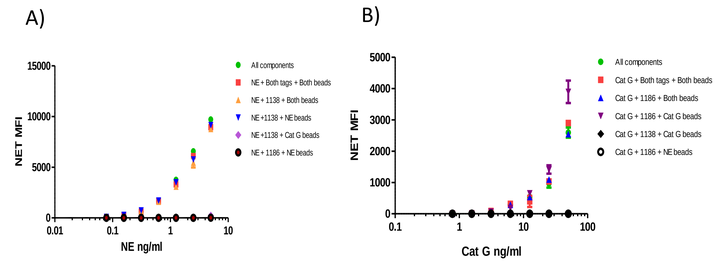

Figure 2. Experiments assessing NSB between A) NE and key Cat G reagents and B) Cat G and key NE reagents

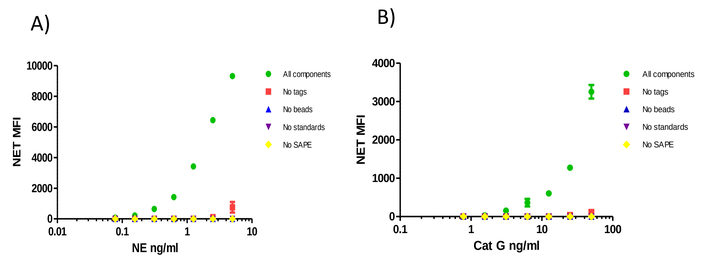

Figure 3. Omission assays to pinpoint other possible sources of NSB when multiplexing for A) NE and B) Cat G

Discussion and Conclusion

The multiplexing of NE and Cat G offers considerable advantages over existing monoplex assays including faster data acquisition and reduced sample volume requirements; with its higher sensitivity also raising the possibility of measuring the active form of these proteases in other sample types, such as plasma.