Validation of a multiplex assay for measurement of IL-6, IL-8, and TNF-α in sputum

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Introduction

The pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-alpha (TNF-α), are strongly associated with the 'cytokine storm', an overreactive immune response that often progresses to systemic sepsis and death[1]. Unfortunately, this biological complication is often observed in severe Covid-19 disease, with elevated plasma levels of IL-6, IL-8, and TNF-α a defining feature[2]. However, as Covid-19 targets the pulmonary epithelium, a multiplex assay, that measures key cytokines in sputum, may assist in patient stratification.

Materials & Methods

Sputum samples (n=40), obtained from a research sample biobank, were used for validation of a multi-analyte kit for measurement of IL-6, IL-8, and TNF-α (Bio-techne). Sample processing involved an initial x 5 dilution in PBS, centrifugation at 3,000 g for 30 minutes at 4°C, followed by aliquoting and storage of sputum sol at -80°C.

Sample matrix interference was first evaluated with ten sputum sol samples, per cytokine, analysed across two lots of the multi-analyte kit, at dilutions ranging from neat up to x200. Corrected cytokine levels were then compared across dilution factors to identify the minimum required dilution (MRD).

Assay selectivity and dilutional linearity were also evaluated. For selectivity, four samples per analyte were spiked +/- the analyte under examination; with linearity assessed by a subsequent x2 serial dilution of each spiked sample.

Next, cross-reactivity of the multi-analyte kit was assessed by spiking sputum sol samples with two non-target cytokines. For instance, during IL-6 testing, samples were screened +/- IL-8 and TNF-α. Spiking concentrations were 500 pg/ml, 1000 pg/ml and 250 pg/ml for IL-6, IL-8 and TNF-α, respectively.

To ensure quantified cytokine concentrations are accurate and reproducible, inter and intra assay precision/coefficients of variation (CVs) must be determined. For inter assay CVs, eight samples were measured, in duplicate, on six analytical runs, with experiments performed across two lots of the multi-analyte kit. For intra assay CVs, three samples, across the range of the standard curve, were tested (10 replicates), on one analytical run.

References

1. Tisoncik, J. (2012) *Microbiology and Molecular Biology Reviews*, 76 (1): 16-32
2. Del Valle, D. (2020) *Nature Medicine*, 26: 1636-1643

Results

Matrix interference is inferred by a linear relationship between corrected analyte concentrations and increasing dilution factor. Negligible interference was observed during analysis of IL-6 and IL-8 (Fig. 1A). In contrast, a x2 dilution was required to attenuate the matrix effect noted during TNF-α measurement (Fig. 1B).

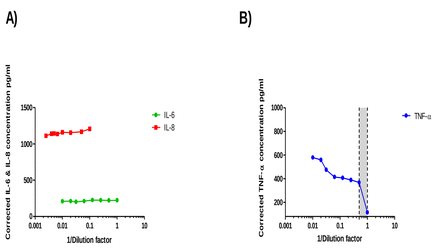

Figure 1. Matrix assessment of **A)** IL-6 and IL-8 and **B)** TNF-α. Grey-shaded region denotes the dilution range that attenuated matrix interference.

Selectivity was calculated to fall within the 80-120% range for all three cytokines. Moreover, excellent linearity was observed, over a range of dilutions, for each analyte (Table. 1); thereby, illustrating the assay's flexibility to analyse samples that contain varying levels of the target analytes.

Analyte	Selectivity (%)	Dilutional linearity
IL-6	112.7	102.6
IL-8	90.4	106.1
TNF-α	113.4	113.3

Table 1. Assessment of selectivity and dilutional linearity.

Regarding cross-reactivity, no significant difference was detected between cytokine levels at baseline and in spiked samples, thereby highlighting the excellent specificity offered by this multi-analyte kit (Fig.2A-C).

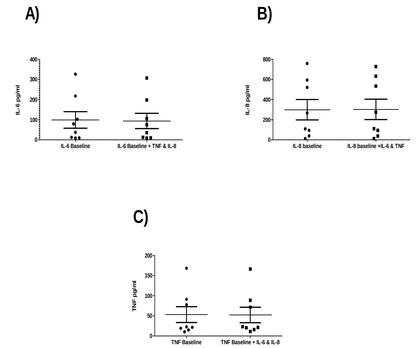

Figure 2. Cross-reactivity analysis for measurement of **A)** IL-6, **B)** IL-8 and **C)** TNF-α

Intra and Inter-assay CVs were determined to fall below the desired targets of <10% and <20%, respectively (Table 2).

Analyte	Inter assay CVs (%)	Intra assay CVs (%)
IL-6	10.85	3.61
IL-8	10.86	2.03
TNF-α	11.82	3.28

Table 2. Inter and Intra assay CVs.

Discussion and Conclusion

The data collected, during this validation study in our laboratory, highlights the suitability of this multi-analyte kit for quantification, of IL-6, IL-8 and TNF-α, in sputum sol samples. Further work is required to confirm whether this cytokine panel has specific utility in identifying those at greatest risk of developing severe Covid-19 disease.