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Electronic Supplementary Information (ESI) for

Multiplexed Femtomolar Quantitation of Human Cytokines in a Fluoropolymer Microcapillary Film

Ana P. Castanheira,[a] Ana I. Barbosa,[b] Alexander D. Edwards[ac] and Nuno M. Reis*[ab]

[a] Capillary Film Technology Ltd, West Sussex RH14 9SJ, United Kingdom

[b] Department of Chemical Engineering, Loughborough University, Leicestershire LE11 3TU, United Kingdom. E-mail: n.m.reis@lboro.ac.uk; Tel: +44 (0)1509 222505; Fax: +44 (0)1509 223923

[c] Reading School of Pharmacy, University of Reading, Reading RG6 6AD, United Kingdom. E-mail: a.d.edwards@reading.ac.uk; Fax: +44 (0)118 931 4404; Tel: +44 (0)118 378 4253

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SUPPLEMENTARY EXPERIMENTAL DESIGN

Multi-syringe aspirator (MSA) device.

This was first presented by Barbosa *et al,.*[1] and consists of a semi-disposable device containing an array of 8, 1 ml plastic syringes that is interfaced with the pre-coated MCF strips using push fit seals and a customised sample well, as shown in Fig. S1.

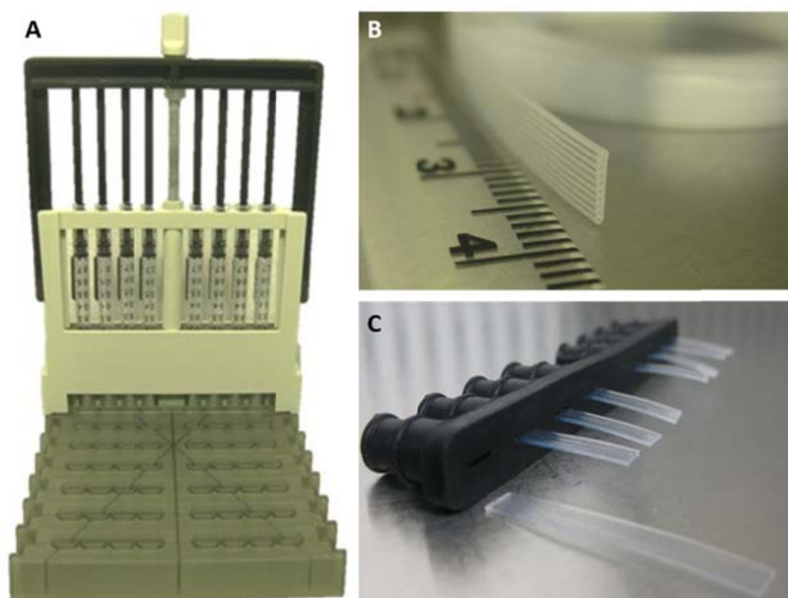


Fig. S1 Overview of Multi-Syringe Aspirator (MSA) device and miniaturised fluoropolymer MCF platform. a) MSA device capable of doing 80 simultaneous tests using an array of 8 disposable 1ml syringes and pre-coated 30 mm fluoropolymer MCF strips. b) Fluoropolymer MicroCapillary Film (MCF). c) Push-fit seals holding 8 cytokine test strips

Singleplex IL-1β Immunoassay Optimisation

Several parameters were tested in order to optimise and improve the sensitivity of cytokines detection and quantification in the MCF based on colorimetric ELISA quantitation using a flatbed scanner. Because of the significant differences in the volume of reagents, surface-area-to-volume (SAV) ratio and light path distance in small capillaries compared to 96-microtiter plate well, all immunoassay conditions had to be optimised in order to produce a colorimetric signal that could be detected using a flatbed scanner. Table S1 summarises the full range for variables tested and shortlisted from an extensive initial screening. All parameter were optimised in respect to maximum signal-to-noise ratio or signal-to-background ratio.

The optimum capAb concentration was determined by coating in duplicate 8 strips of 3 cm length with 0, 10, 20 and 40 µg/ml of IL-1β mAb. For the control (0 µg/ml of capAb) the strip was incubated with PBS only. On a separate experimental set, detAb was tested in duplicate at a concentration of 0, 5, 10 and 20 µg/ml using MCF strips coated with 10 µg/ml or 20 µg/ml of IL-1β capAb. On both experimental sets, the concentration of IL-1β recombinant protein used was 200 pg/ml. To test the performance of different blocking solutions, two 12 cm long MCF strips were coated with 10 µg/ml of IL-1β capAb. Following 2 hours of incubation, one of the strips was blocked using Bovine Serum Albumin 1% (BSA) and the second one with 2.5% of Fetal Bovine Serum (FBS) and 0.02% of Sodium Azide (NaN_3). The results were then analysed in respect to the initial rate of absorbance generation. During the optimisation of capAb and detAb concentrations and blocking solutions ExtrAvidin was used as enzyme.

Table S1. Optimised assay conditions for human IL-1β singleplex measurement

Unit Operation	Method Parameters	Ranges or type
capAb	Incubation time	overnight 2 hours
	concentration	20 µg/ml
Blocking solution	Incubation time	1 hour
Recombinant protein	Incubation time	30 min
detAb	concentration	10 µg/ml
Enzyme	type	EA-HRP HSS-HRP HSN-HRP

It is important to mention that a washing step before the antigen incubation was crucial for the good performance and reduced variability of the assay. During preliminary assay optimization the two approaches were tested and it was verified that the washing step between the blocking and the addition of the antigen was very important (Figure S2).

Fig. S2 Cytokine test strips imaged after OPD conversion, showing the relevance of washing before addition of antigen. The strips were incubated with decreasing concentration of antigens from the left to right hand side of the picture.

Selection of enzymatic amplification system

Peroxidase is a popular enzyme in ELISA for presenting a very high turnover number, therefore a range of polymerized and conjugated peroxidase enzymes was tested. For that purpose, a given length of MCF was directly coated with 2 µg/ml of biotinylated detAb following similar procedure to the capAb coating. The MCF was then trimmed in short strips and loading with either High Sensitivity NeutrAvidin–HRP (HSN-HRP) or High Sensitivity Streptavidin–HRP (HSS-HRP). Each enzyme was tested in duplicate and one control strip (without enzyme solution) was also prepared for each enzyme tested at each given concentration. HSN-HRP and HSS-HRP were tested for a concentration of 4, 2, 0.2 and 0.1 µg/ml and compared with the results obtained with 4 µg/ml of ExtrAvidin. Performance was then compared in respect to total signal intensity, background intensity and initial rate of substrate conversion. High Sensitivity Streptavidin-HRP revealed increased signal and substrate conversion rate compared to High Sensitivity NeutrAvidin-HRP and ExtrAvidin (Fig. S3). For the tested, Avidin, Streptavidin and NeutrAvidin each were reported by the manufacturer to bind four biotins per molecule with high affinity and selectivity.[2] Piran, *et al*[2] measured the dissociation rate constants of the biotin/streptavidin and biotin/egg avidin complexes and found that the dissociation rate constant for streptavidin (2.4×10^{-6} s^{-1}) was about 30 times faster than that observed for biotin from avidin (7.5×10^{-8}), which can explain the higher signal obtained with HSS-HRP in a short period of time.

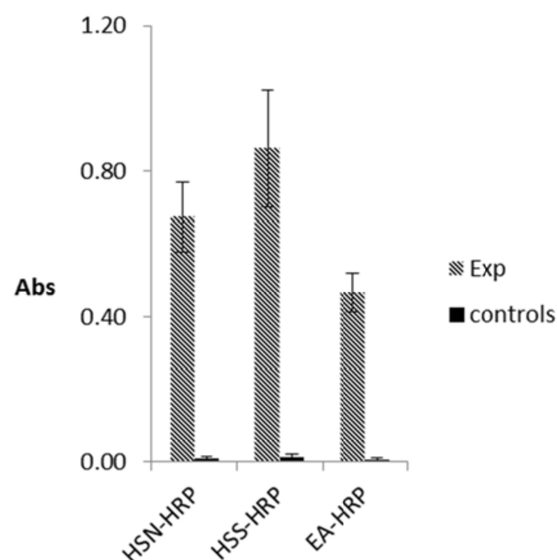


Fig. S3 Comparison of different Peroxide enzymes tested for colorimetric amplification: High Sensitivity NeutrAvidin-HRP (HSN-HRP), High Sensitivity Streptavidin-HRP (HSS-HRP) and ExtrAvidin-HRP (EA-HRP). The enzymes were compared at same concentration of 4 μg/ml

Assay Variability

The assay sensitivity was assessed as LoD or "Cut-off value" of the assay, which was defined by the level above which samples were considered positive, and determined as the mean Absorbance (Abs) of the negative control plus three times the standard deviation of the blank samples. The Limit of Quantitation (LoQ) was also determined for all the cytokines tested using the method described by Ederveen,[3] which uses the best combination of %Precision and %Accuracy between the experimental and expected values calculated using a calibration curve. For each experiment, the expected values were calculated and an experimental versus expected values plot was drawn to find the linear range. From this, the accuracy and precision were then calculated. The LoQ was defined as the concentration corresponding to the Abs that revealed the first value in which the Accuracy was between 80-120% and Precision <20%.

The linear range was defined as the interval between the lower and the upper concentration of recombinant protein (IL-1β) for which an appropriate level of precision, accuracy and linearity was obtained. The precision is herein referred to as Intra and Inter-assay precision. To determine the Intra-assay precision three samples with different concentrations (near the Lower Limit of Quantification, LLoQ, Middle range, MR; and near the Higher Limit of Quantification, HLoQ,

all determined from the calibration curve) were tested in six replicates (6 strips with 10 capillaries; 60 capillaries in total). The average Abs values and the standard deviation for each sample were calculated and the Coefficient of Variation (CV) was also determined for each concentration within a given assay run. The Inter-Assay Calibration was determined by running three assays in different days and using different MSA devices, in which samples were analysed in duplicate, using the same three concentrations of recombinant proteins (corresponding to LLoQ, MR and HLoQ). The average and the standard deviation for each sample were calculated and the CV determined for each concentration between the assay runs. Typical CVs for ELISA in 96-well MTP are in the range of 10–20%. [3,4]

Accuracy or Recovery was also determined from the calibration curve by comparing the expected value with the actual cytokine concentration in the assay. The expected versus the average of the measured values was determined for each sample, by calculating the %Recovery= assay value/expected value x 100. The typical range for accepted accuracies is 80-120%.[3]

Qualitative Duplex Assay

In order to demonstrate the ability of the new miniaturized platform to detect simultaneously more than one cytokine a simple qualitative duplex assay was developed with of IL-1β and IL-6 reagents. For this purpose, solutions containing IL-1β or IL-6 capAb were injected into each individual capillary using a small syringe needle. The MCF strip was then incubated for 2 hours at room temperature, and then further incubated for 1.5 h with the blocking solution and washed with PBS-T. The strips were then trimmed into 30 mm long individual test strips and attached onto the MSA. Equal concentration of recombinant proteins (0.5 ng/ml) and detAb (10 μg/ml) were then used. All subsequent steps followed same sandwich ELISA procedure described in the main manuscript.

Quantitative Triplex Assay

A quantitative triplex assay consisting of full response curve for each cytokine was performed for simultaneous quantitation of IL-1β, IL-12 and TNFα. Individual capillaries on a 25 cm long fluoropolymer MFC strip (containing 10 capillaries) were injected and incubated into one pair of capillaries each with one of the following solutions: PBS (overall negative control), 3% BSA (blocking solution control) or IL-1β, IL-12 capAb or TNFα at 20 μg/ml. All subsequent ELISA

steps were as already described for the singleplex and duplex assays, with the exception that standard curves were prepared using a 1:3 dilution series of recombinant protein. All recombinant proteins and detAb solutions for each cytokine were combined at same concentration, which ultimately represents 3 times higher protein content on each solution well when compared to singleplex detection. Combining different biotinylated detAb for multiplex ELISA detection was found to significantly affect the individual cytokine performance by increasing the background, therefore it was necessary to re-optimise the multiplex assay, in respect to detAb and enzyme concentration, to maintain similar signal-to-noise ratios to singleplex assays. This is described in Supplementary Results section.

SUPPLEMENTARY RESULTS

Assay Optimisation

The first two parameters tested were the incubation times of the capAb and recombinant proteins. Two hours incubation at room temperature was sufficient to fully immobilize the capAb by passive adsorption, as no differences were detected in signal strength and signal-to-noise ratio (Fig. S4a). This had the advantage of saving the typical overnight incubation required for MTP sandwich ELISAs. Equally, the incubation of recombinant protein for sensitive detection could be reduced to 30 min in the fluoropolymer MCF without compromising sensitivity (Fig. S4b). This is linked to the very short diffusion distances in the plastic microcapillaries. A parameter found paramount in controlling the signal-to-noise ratio in the fluoropolymer MCF ELISA was the blocking solution, for that reason few different formulations were tested. BSA and FBS are commonly used for blocking non-specific binding sites in plastic surfaces. Although no significant difference could be detected in respect to Abs signal intensity, the kinetic analysis of the OPD conversion in the capillaries for different cytokine concentrations revealed poor performance for both 1% BSA or 2.5% FBS in respect of background development. Fig. S4c shows the initial rates of assay; in that plot the initial velocity v_0 corresponded to the rate of generation of absorbance in the MCF during the first few minutes of OPD conversion in the full cytokine sandwich ELISA. BSA and FBS have similar proteins in size and molar rations on their composition, since BSA is the main compound present in FBS; however on both cases a high background was detected ($Abs_0 \approx 0.05$). In order to reduce the background which directly controls

to the sensitivity of the assays, a synthetic SuperBlock blocking solution from Thermo Scientific was tested, which revealed lower backgrounds ($Abs_0 \approx 0.02$) (data not shown).

The use of higher capAb concentrations of 40 µg/ml and above resulted in increased background and reduced signal (Fig. S4d), suggesting FEP antibody adsorption and/or orientation was not favored by the presence of a very high capAb concentration. This is presumably linked to the orientation of the surface adsorbed capAb molecules.[5]

The effect of detAb concentration was tested for a range between 0 and 20 µg/ml, and it was also observed a benefit in using 10 µg/ml (Fig. S4e). Again, this is significantly higher than the concentrations normally used for sensitive sandwich ELISA in MTPs and the increase in the signal can be explained based on the same binding equilibrium principle. A large solution excess of detAb favors the formation of the complex capAb-Ag-detAb at the surface of the plastic capillaries, which is linked to the larger SAV ratio in small bore microcapillaries.

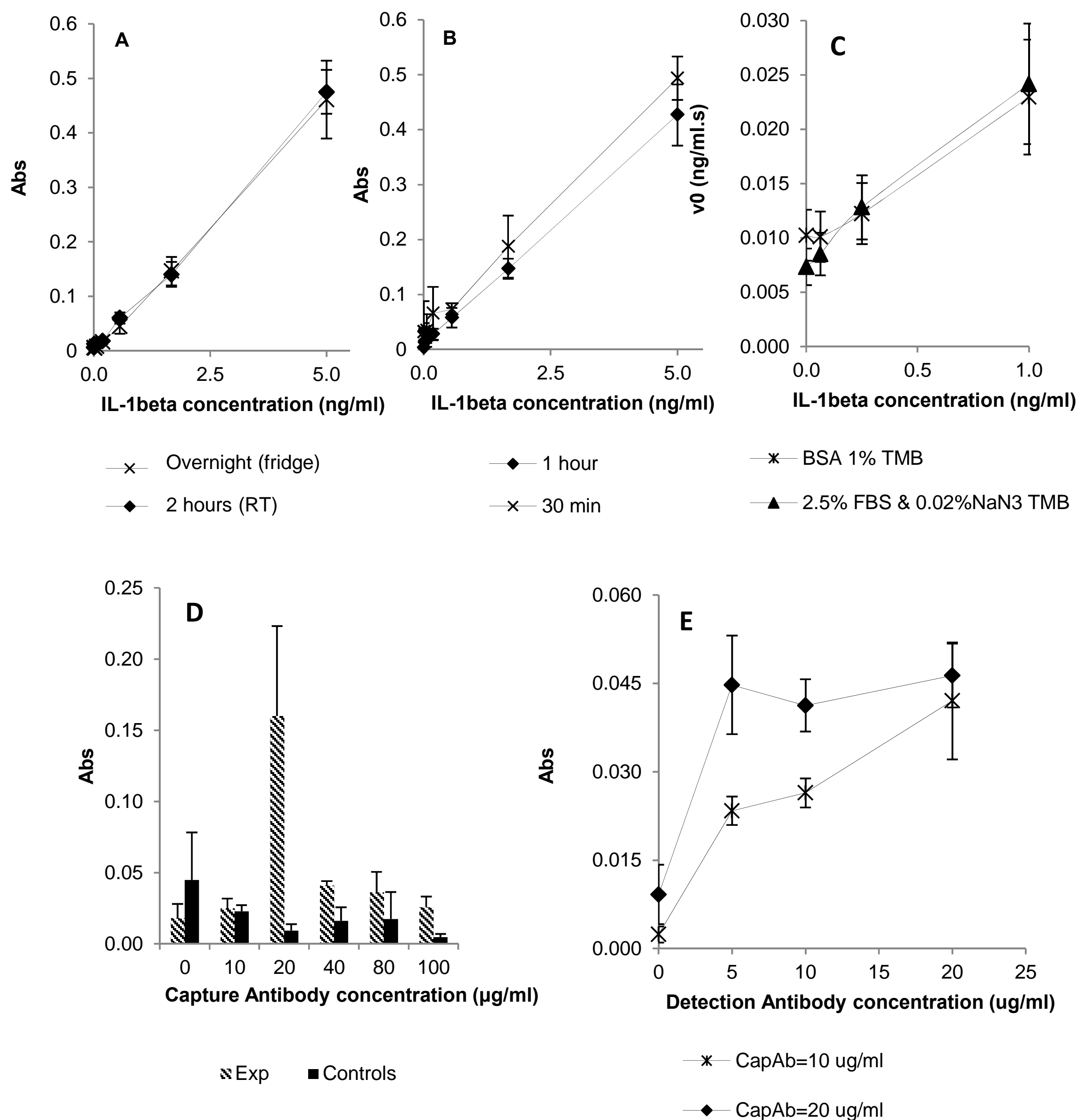


Fig. S4 Optimisation of IL-1β cytokine immunoassay measurement in the fluoropolymer MCF. a) and b) show effect of of incubation time of capAb and recombinant protein, respectively. c) Initial rates of colourimetric signal generation in the MCF strips for different blocking solutions (BSA 1% and FBS 2.5%). d) Effect of capAb concentration (20 μg/ml). e) Effect of detAb concentration for two different capAb concentration coatings. Assays conditions are detailed in

Experimental Design section in the manuscript. The optimised concentrations for capAb and detAb were considered 20 µg/ml and 10 µg/ml, respectively

Limit of Quantitation of the Colorimetric Detection Device

A series of dilutions of 2,3-diaminophenazine (DAP), the final product of the conversion of the substrate OPD by the immunoassay enzyme HRP, were scanned in MCF with a flatbed scanner starting at a concentration of 2 mg/ml, and in parallel peak absorbance (450 nm) of the same dilutions was measured in a 96-well MTP using a microplate reader (Fig. S5). The DAP absorbance in the blue channel of the scanned image was calculated by image analysis.

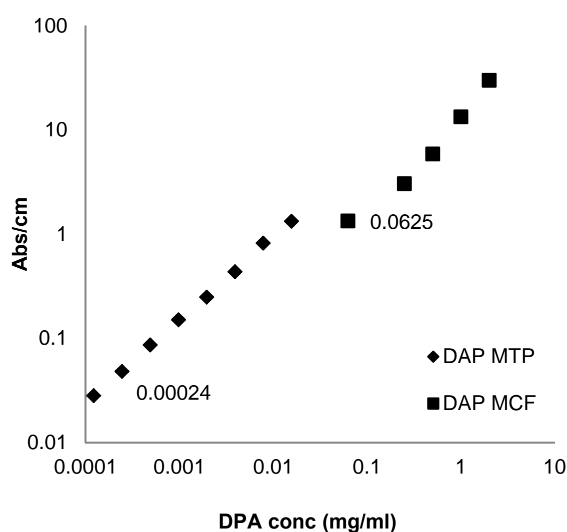
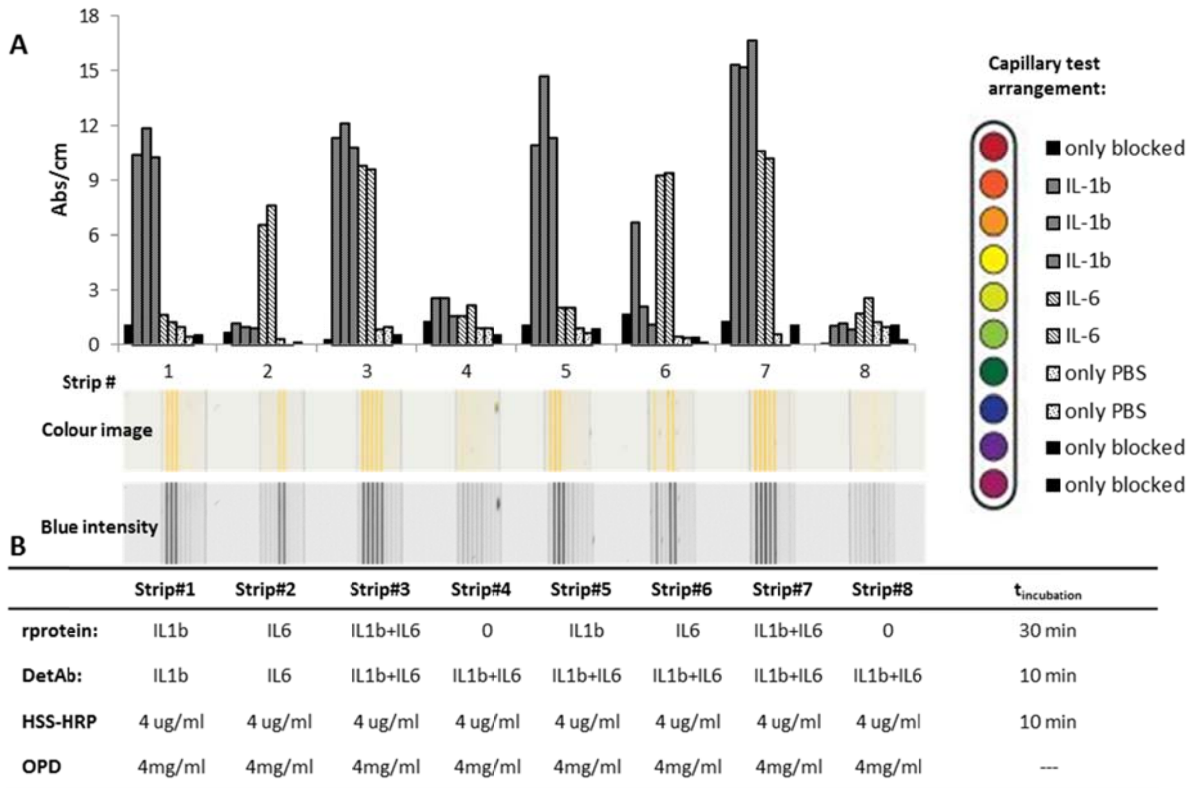


Fig. S5 Response curves for DAP detection in the MCF with flatbed scanner and 96 well MTP with microplate reader (450 nm). Only concentrations corresponding to the range of DAP concentration versus normalised Abs are presented for the MTP and fluoropolymer MCF

Qualitative Duplex Detection

To demonstrate the capability of simultaneous detection of two or more cytokines on each fluoropolymer MCF strip, a duplex qualitative assay using IL-1β and IL-6 was performed and analysed. All capillaries showed a positive color signal according to the capAb coating pattern (Fig. S6), which confirms the possibility of detecting simultaneously more than one cytokine from a single sample. The main assay conditions used on each MCF strip in the 8-channel MSA device are shown in Fig. S6b. The higher signal observed for IL-1β cytokine was possibly due to

the fact that the optimisation was based on this cytokine, which led to IL-6 underperforming. The background values were in general very low with the exception of a single capillary in strip number 6 which showed increased noise, and the signal-to-noise ratio (SNR) in the order of 5.



	Strip#1	Strip#2	Strip#3	Strip#4	Strip#5	Strip#6	Strip#7	Strip#8	$t_{incubation}$
rprotein:	IL1b	IL6	IL1b+IL6	0	IL1b	IL6	IL1b+IL6	0	30 min
DetAb:	IL1b	IL6	IL1b+IL6	IL1b+IL6	IL1b+IL6	IL1b+IL6	IL1b+IL6	IL1b+IL6	10 min
HSS-HRP	4 ug/ml	4 ug/ml	4 ug/ml	4 ug/ml	4 ug/ml	4 ug/ml	4 ug/ml	4 ug/ml	10 min
OPD	4mg/ml	4mg/ml	4mg/ml	4mg/ml	4mg/ml	4mg/ml	4mg/ml	4mg/ml	---

Fig. S6 Qualitative duplex assay for detection of IL-1β and IL-6 cytokines. a) Capillaries 2, 3 and 4 were coated with Anti-Human IL-1β antibody purified; Capillaries 5 and 6 were coated with Anti-Human IL-6 antibody purified; Capillaries 7 and 8 were incubated only with PBS and capillaries 1, 9 and 10 were only blocked for 1h30min (i.e. not coated with capAb). In strips 1 and 5 only 0.5 ng/ml of IL-1β recombinant protein was added, and strips 2 and 6 only 0.5 ng/ml of IL-6 recombinant protein used. Strips 3 and 7 were filled with both recombinant proteins at the same concentration and the strips 4 and 8 were used as control strips (i.e. with 0 ng/ml of recombinant protein). b) Duplex assay details. The assay conditions are detailed in Supplementary Experimental Design section

Optimisation of the Triplex Assay

In order to reduce the background, the triplex assay was optimised by testing different conditions of detAb and Enzyme. A MCF strip was coated with 20 µg/ml of capAb of each cytokine

following the procedure described for quantitative triplex assay in Supplementary Experimental Design section. Duplicated strips plus a blank strip (with no added recombinant protein) were tested for the different combinations of 2.5, 5 and 10 μg/ml of detAb and 1, 2 and 4 μg/ml of Enzyme, using 333 pg/ml of recombinant protein (which fitted the linear range of all there cytokines tested). Several full response curves were built using the detAb and Enzyme concentrations giving the higher signal-to-noise ratio. The optimised conditions were 10 μg/ml detAb and 4 μg/ml HSS-HRP (Fig. S7).

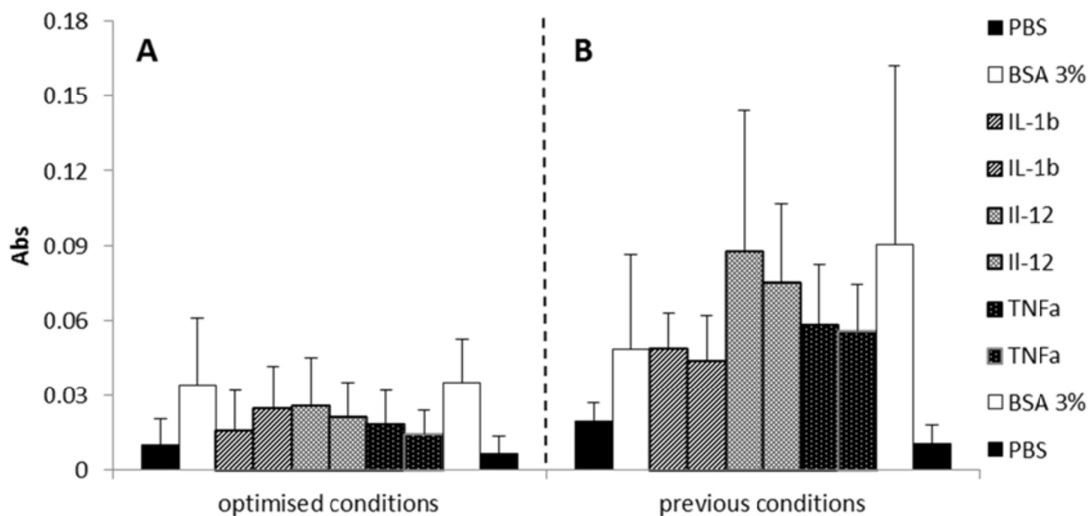


Fig. S7 Comparison of background in Triplex Cytokine Assay using optimised a) and initial b) assay conditions. a) 10 μg/ml detAb and 4 μg/ml HSS-HRP and b) 5 μg/ml detAb and 2 μg/ml HSS-HRP

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