AnteoTech

Proof of Concept

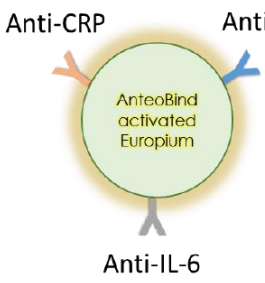
Sepsis Biomarkers Full-strip Lateral Flow Assay Development Using AnteoBind™ Activated Europium Particles and EuGeni™

Background

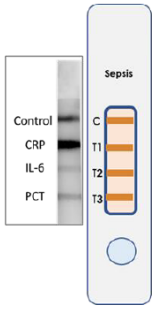
According to the World Health Organisation up to 30 million people worldwide are affected by sepsis, potentially leading to 6 million deaths per year (World Health Organisation, 2020). Clinically diagnosed as the presence of acute infection with organ dysfunction, it is presumably the result of an out-of-control immune response due to underlying infections. Even if sepsis does not result in death, it can create lifelong disabilities in survivors, with 40% of cases occurring in children under five (University of Pittsburgh, 2020). Once the condition is present in the body illness progresses rapidly, decreasing the time available to provide accurate diagnosis and treatment. Early onset detection is difficult and most accurately determined by a panel of different tests. The trend of sepsis diagnosis is moving towards rapid Point-of-Care testing, though one major issue involves the development of a quantitative multi-analyte test.

Our Approach

Utilising AnteoBind activated europium particles, AnteoTech aimed to prove a concept for sepsis rapid diagnostic screening. The goal was to develop a lateral flow test strip capable of detecting three different analytes and produce results of clinical relevance. This was achieved by developing a high sensitivity, quantitative lateral flow immunoassay (LFIA) to be used with EuGeni to detect sepsis-related analytes.



Conjugate Design



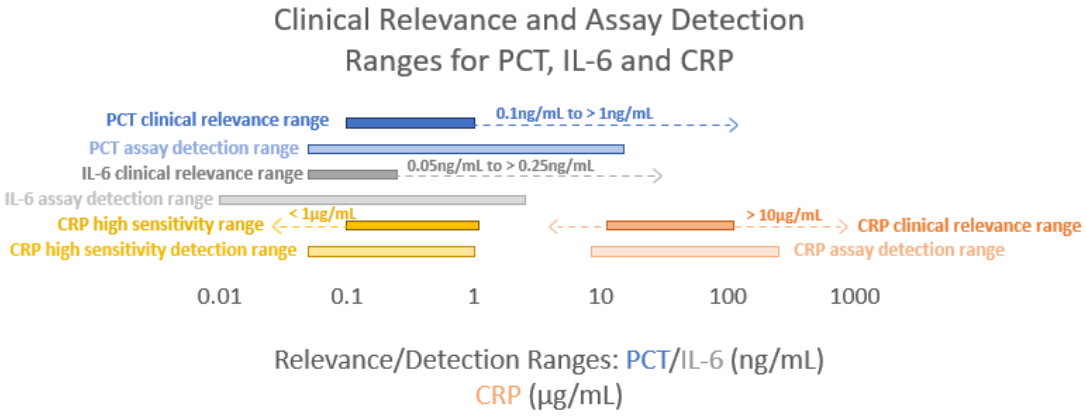
Strip Design

Key Achievements

By combining the strength and flexibility of AnteoBind activated europium particles with the sensitivity of the EuGeni AX-2X-S reader, AnteoTech demonstrates success in Proof of Concept for a multi-analyte sepsis rapid diagnostic test indicating a commercially viable option for product development.

- Multi-functional europium conjugates were prepared by co-conjugating a mixture of anti-PCT (procalcitonin), anti-IL-6 (Interleukin-6) and anti-CRP (C-reactive protein) antibodies to a single AnteoBind™ coated particle, allowing easier multi-plex assay design by eliminating the need to balance particle ratios for each individual analyte, while minimising background fluorescence related to the use of more particles. strip with conjugate release pad and lateral flow membrane striped with 3 test lines (to detect PCT, IL-6 and CRP) and a control line were constructed successfully.
- Full strip with conjugate release pad and lateral flow membrane striped with 3 test lines (to detect PCT, IL-6 and CRP) and a control line were constructed successfully.

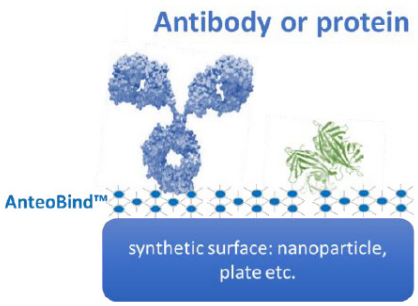
- Using a sample volume of 100µL antigen spiked in 50% human serum the assay detection range for both PCT (15ng/mL to 50pg/mL) and IL-6 (2.5ng/mL to 10pg/mL) were within the clinical relevance ranges established in previous studies: 0.1ng/mL to ≥ 1ng/mL for PCT and 0.05ng/mL to ≥ 0.25ng/mL for IL-6.
- A competitive effect was observed in the clinical relevance range for CRP (signal peaking at 7.5ng/mL), while the expected titration was observed again from 1ng/mL to 50pg/mL. CRP is detectable at this current stage, though not as clear cut as PCT and IL-6. With further development this CRP component is expected to meet both the clinical relevance criteria while also hitting the high sensitivity testing requirements. The results obtained show a solid starting point for further optimisation on the detection of analytes. Further work is needed to take this proven concept to commercialisation.



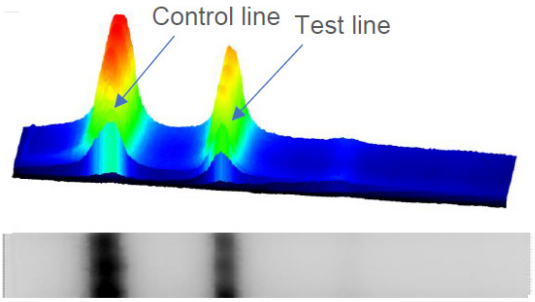
The minimum requirements for clinical testing ranges are shown above, with bars displaying the ideal testing ranges and arrows denoting analyte levels seen during various stages of sepsis progression. In the case of the CRP high sensitivity range the blanket requirement is detecting anything less than 1µg/mL

Key Features

- Three different antibodies on one particle: AnteoBind allows for the simultaneous attachment of multiple, different proteins to the europium particle, creating a multi-functional nanoparticle capable of detecting all three sepsis analytes. Less particles are needed for each multiplex assay, lowering the level of cross-reactivity.
- Europium particles and fluorescence-based assays offer a significant improvement (e.g. 7 to 300-fold) (Juntunen, E. et al., 2012) in sensitivity compared to conventional gold-based colourimetric assays, allowing the detection of analytes using a lower sample volume.



- The EuGeni AX-2X-S instrument is a portable, highly flexible, and easy to use rapid testing instrument platform designed to provide quantitative results for multiplexed fluorescent immunoassays. The instrument can be operated in Standalone mode or in Kinetic Designer mode via connected laptop.
- Three analytes on one strip: a Triplex assay tuned with low cross-reactivity and high specificity in a single cartridge, lateral flow strip format allows for easier user handling and simplifies the assay process

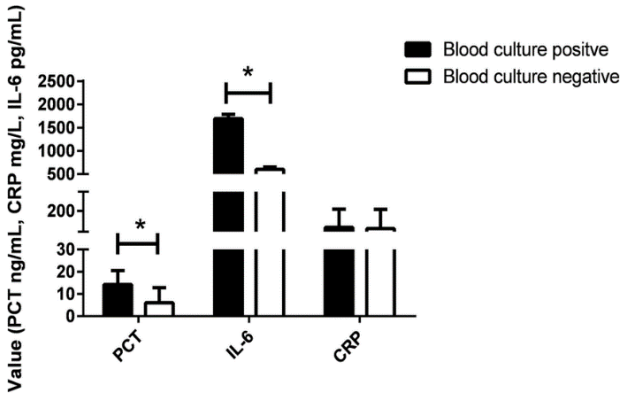


AX-2X-S strip visualization

Chosen Analytes

Due to difficulties in differentiating sepsis from other acute inflammatory responses a variety of sepsis-related biomarkers are screened so that the appropriate treatment can be applied. In previous studies, biomarkers such as procalcitonin (PCT), interleukin 6 (IL-6) and interleukin 8 (IL-8) have been proposed as promising candidates for sepsis determination, in addition to the conventional testing of C-reactive protein (CRP) and lactate levels as well as leukocyte counts (Faix, J.D., 2013). PCT, IL-6 and CRP were used as the biomarkers for the development of AnteoTech's sepsis test. Several references were selected and described below:

- PCT - pre-cursor to calcitonin. Levels rise in response to bacterial infection and tissue injury though typically see a significant increase during systemic bacterial infection and sepsis (Yunus et al., 2018). Listed as a diagnostic criteria for sepsis as part of the Surviving Sepsis Campaign (Dellinger et al., 2008).
- IL-6 - an initial response cytokine released in response to trauma. Whilst not specific to sepsis, IL-6 production is seen to increase during sepsis and levels are significantly higher in those experiencing septic shock than those without. Amongst the cytokines produced during sepsis, plasma IL-6 displayed with best correlation with mortality rates (Chaudry et al., 2013).
- CRP - a non-specific marker for infection and inflammation, significant and rapid increases in CRP can be observed during the early onset of sepsis and fall quickly with successful treatment (Faix, J.D., 2013). Listed as a diagnostic criteria for sepsis as part of the Surviving Sepsis Campaign.



Plot of procalcitonin (PCT), C-reactive protein (CRP), and interleukin 6 (IL-6) plasma concentrations in blood culture–positive and blood culture–negative groups. Significant differences (indicating as asterisk) in PCT and IL-6 were observed between groups. (Wu, Q. et al., 2018)

PCT LEVELS (ng/ml)	Sample Size	IL-6 (pg/ml) Mean±SD	CRP (mg/l) Mean±SD
<0.5 (local infection)	7	11.78±5.34	27.43±17.69
0.5-1.9 (SIRS)	6	9.54±5.5	43.33±22.51
2-10 (sepsis)	10	27.59±14.3	73.5±24.73
>10 (severe sepsis)	57	94.82±78.21	124.3±39.67

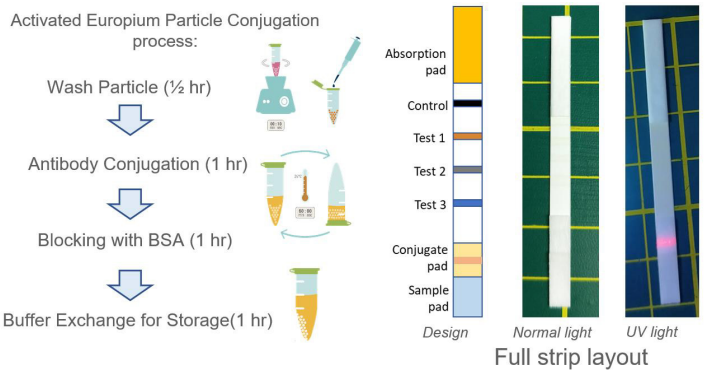
Distribution of IL-6 and CRP levels as per the PCT values (Sharma, S. & Duggal, N., 2019)

Preliminary Evaluation

Conjugates for detecting a single analyte were prepared and tested using buffers spiked with antigen in a half strip format. The antigen range tested included 1ng/mL down to 20pg/mL to establish an estimate for the overall single analyte assay sensitivity. Multi-functional co-conjugates were then prepared and tested in the same format, with changes being made to the antibody ratios to provide equivalent results to the single antibody conjugates. From this step the particles were dried down and assembled in a full strip with further optimisation being made to account for serum testing. A brief outline of the conjugate, strip and assay preparations steps are included in the next section.

Triplex Conjugate Optimisation

AnteoBind Particle-Conjugation kit reagents were used to conjugate anti-PCT, anti-IL-6 and anti-CRP antibodies to the AnteoBind pre-activated 300nm Estapor® europium nanoparticles. A protein mixture containing the three antibodies (at a designed ratio) was prepared and to which washed pre-activated europium particles were added. The particle mixture was incubated for one hour on an end-over-end rotator before BSA blocking solution was added. After an additional hour of mixing the particles were washed and stored in storage solution, ready to be dried down on to a conjugate release pad.



Full Strip Preparation

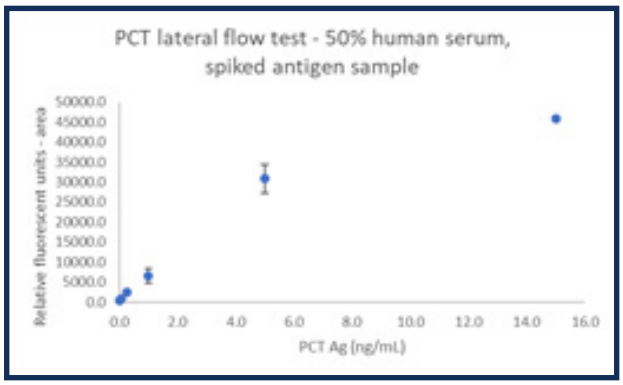
Glass fiber conjugate pad was treated with a formulated pre-treatment solution before the multi-functional europium particles were prepared in drying buffer and striped across the conjugate pad. The conjugate pad dried overnight before being assembled in the full strip. The particle load chosen was 0.12µg of particles per strip. Sartorius UniSart CN 95 lateral flow membranes were striped with a goat anti-mouse control line, anti-CRP test line, anti-IL-6 test line and anti-PCT test line. All lines were prepared in a striping buffer containing BSA. The full card was assembled with overlaps being applied across the sample pad, conjugate pad, and membrane. The absorption pad also overlapped the top section of the membrane. The card was cut into 4mm widths and allowed to dry completely before testing. The results obtained for both PCT and IL-6 show that the assay is capable of detecting levels of analyte below what is clinically relevant up to levels seen in the moderate progression of sepsis, giving confidence to the claims of achieving clinical relevance and earlier detection of these two markers.

Assay Setup

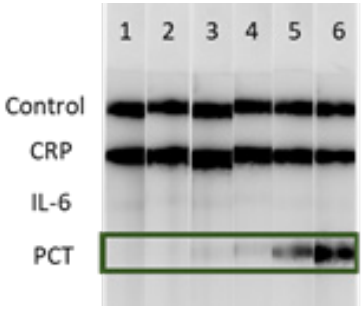
Antigen ranges of clinical relevance were prepared for each individual marker, with PCT and IL-6 antigens prepared in a 50% human serum solution. Due to the high background CRP usually present in human samples CRP samples were prepared in goat serum. 100µL of sample was tested for each antigen point with strips being run in triplicate. Samples were run for 15 minutes before being read in the EuGeni AX-2X-S reader.

Full-strip Results

- Lateral flow test line signals with various antigen concentrations were tested several times and collected by the reader and analysed as shown in the plots and graphs on the right.
- Clinically relevant detection ranges of 15ng/mL to 50pg/mL were achieved for PCT, and 2.5ng/mL to 10pg/mL for IL-6 in strips run in 50% human serum with normal levels of CRP.
- CRP results obtained indicate a competitive response in clinical relevance range, with signals peaking at 10µg/mL in 50% goat serum. Previous testing (20% goat serum) indicates that the ideal range for the current assay sits within the high sensitivity range (< 1µg/mL).

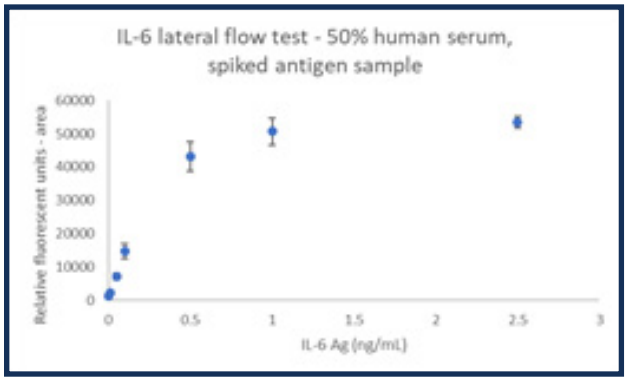


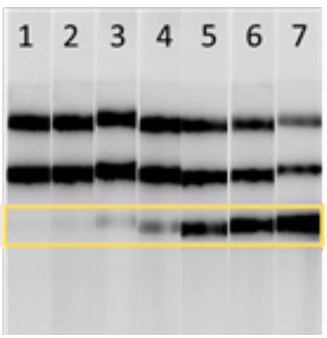
Representative full-strip



PCT Antigen

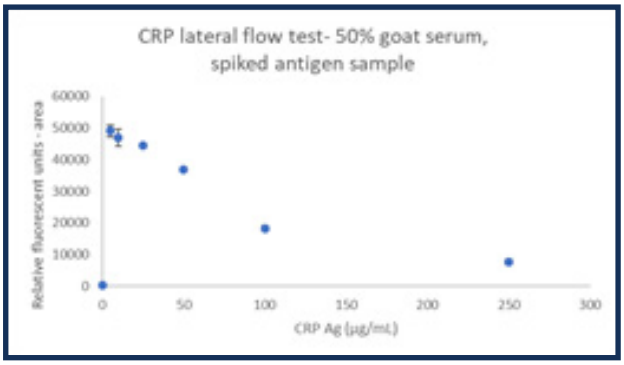
1. 0ng/mL
2. 0.05ng/mL
3. 0.25ng/mL
4. 1ng/mL
5. 5ng/mL
6. 15ng/mL



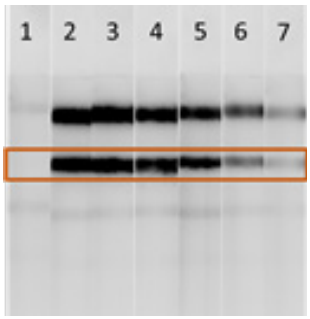


IL-6 Antigen

1. 0ng/mL
2. 0.01ng/mL
3. 0.05ng/mL
4. 0.1ng/mL
5. 0.5ng/mL
6. 1ng/mL
7. 2.5ng/mL

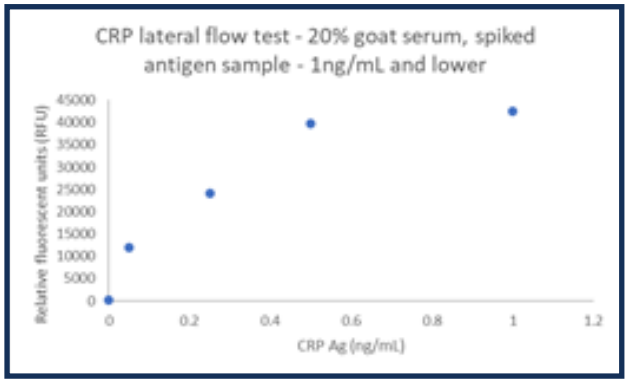


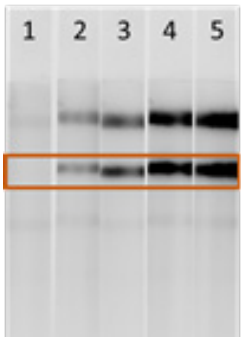
Representative full-strip



CRP Antigen

1. 0ng/mL
2. 5µg/mL
3. 10µg/mL
4. 25µg/mL
5. 50µg/mL
6. 100µg/mL
7. 250µg/mL





CRP Antigen

1. 0µg/mL
2. 0.05µg/mL
3. 0.25µg/mL
4. 0.5µg/mL
5. 1µg/mL

Discussion

The results obtained for both PCT and IL-6 show that the assay is capable of detecting levels of analyte below what is clinically relevant up to levels seen in the moderate progression of sepsis, giving confidence to the claims of achieving clinical relevance and earlier detection of these two markers.

The CRP results indicate that due to the high level of CRP present in the target clinical relevance range (over 1000x higher than PCT and IL-6 ranges) a competition effect is observed, where free CRP in the sample is out-competing the CRP antigen bound to the europium particles, resulting in lower signals at higher antigen levels. This indicates that with adjustment results would be obtainable for range 10µg/mL to 1µg/mL as the detection of CRP in the high sensitivity range providing a promising indication that this is likely.

Conclusion

A Proof of Concept full-strip lateral flow assay for the detection of sepsis markers was achieved, with promising results suggesting that with further work a viable product could be developed.

References

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For further information on the Sepsis Biomarker test contact us at

E sales@anteotech.com **T** +61 7 3219 0085 (Australia)