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White Paper

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Lateral Flow Immunoassay, Multiplexing, StructSure®, Sepsis Diagnostics, Dot-based Dispensing

The Next Generation of LFA - Multiplexing on Unisart StructSure® Membranes

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Abstract

In recent years, multiplex lateral flow assays have become increasingly popular in disease management because they can rapidly analyze multiple parameters or biomarkers simultaneously. Common lateral flow test formats incorporate multiple lines or capture zones on the same test strip, each designed to detect a specific target molecule. There are limitations to these types of in-line tests, including false negatives, cross-reactivities or high antibody consumption. In this white paper, a complete solution including assay development with structured membranes, cassette use, and advanced LFA readout is showcased in a multiplex lateral flow test for the detection of Sepsis biomarkers C-reactive protein (CRP) and Procalcitonin (PCT). The miniaturization of lines to spots using the dot-based dispensing technology from SCIENION GmbH in combination with the separation on the unique structured Unisart StructSure® membrane from Sartorius resulted in improved assay performance and reliability compared to the use of conventional lateral flow tests in line.

Introduction

In the dynamic landscape of healthcare, the demand for swift and accurate diagnostic tools has never been more critical. A poignant example was the COVID-19 pandemic, which revolutionized the understanding of point-of-care testing and highlighted its advantages. Due to their low cost, portability, lack of reagent handling, and simplicity of use by untrained personnel, lateral flow immunoassays (LFA) are especially popular as diagnostic tests. There is, however, much more potential in the LFA test format than the commonly used singleplex strips in line.
Disease treatment requires accurate diagnosis, timely decision-making and effective patient management. However, some diseases require the analysis of multiple biomarkers or analyte levels due to the overlap of symptoms, the insufficient specificity of one marker, or the degree of severity of the disease indicated by the analyte level. Therefore, multiparameter diagnostic tests allow for the simultaneous analysis of multiple factors and provide monitoring of various health conditions through a single test.
Some commercial LFAs combine biomarkers by applying multiple test lines on a stick enhancing diagnostic precision and efficiency (Figure 1 B, left panel). However, this method involves a high consumption of antibodies for the immunological reaction, as well as a high risk for interdependent signals, false negatives, and detector depletion across lines. This white paper presents a method for preventing all of the above-mentioned issues through the miniaturization of lines to spots and their separation using the unique structured membranes Unisart StructSure® from Sartorius.
Follow up for the next generation of LFA multiplexing.

Background

Advantages of Dot-Based LFA

Dot-based LFAs are new in the LFA market segment and open new possibilities for multiplexing and/or quantitation in combination with an automated readout. An enormous saving of capture molecules with the same signal strength is achieved by reducing the amount of capture antibody for immobilization on the solid phase. A common 5 mm LFA stripe consists of one line with approx. 1 µl/cm of 1 mg/ml capture Abs [Parolo, et al. (2020)]. By replacing this line with 3 x 10 nl spot volume (total 30 nl), the amount and cost of expensive Abs can be reduced by approx. 95 % with the same sensitivity. For conventional a-mouse IgG with a price of 200 €/mg, this implies a cost saving of 190,000 € for high-throughput production of 2 Mio sticks per year with SCIENION's sciFLEXARRAYER S100 precision microdispensing systems. In addition, multiple determinations of biomarkers ensure better accuracy and reliability within one test. Another main advantage of using dots for capturing molecules is reducing the risk of false negative signals compared to multiplexing lines. If a particle-conjugated detection antibody is used in an LFA, a wall can be generated by the first line in the running direction due to clogging of the membrane pores by the complex of analytes, capture Abs and detector particles.

As a result, the signal decreases with an increasing number of preceding lines due to the wind shadow effect. This effect is removed by dispensing dots of ≤ 5 nl (Figure 1 A) allowing the combination of various biomarkers and free design of the spot arrangement within a lane or parallel to each other. If the desired sensitivity requires a larger print volume to increase the amount of capture Abs presented, false negative signals can also be avoided by flexible positioning of the spots on the membrane in individual lanes per spot (Figure 1).

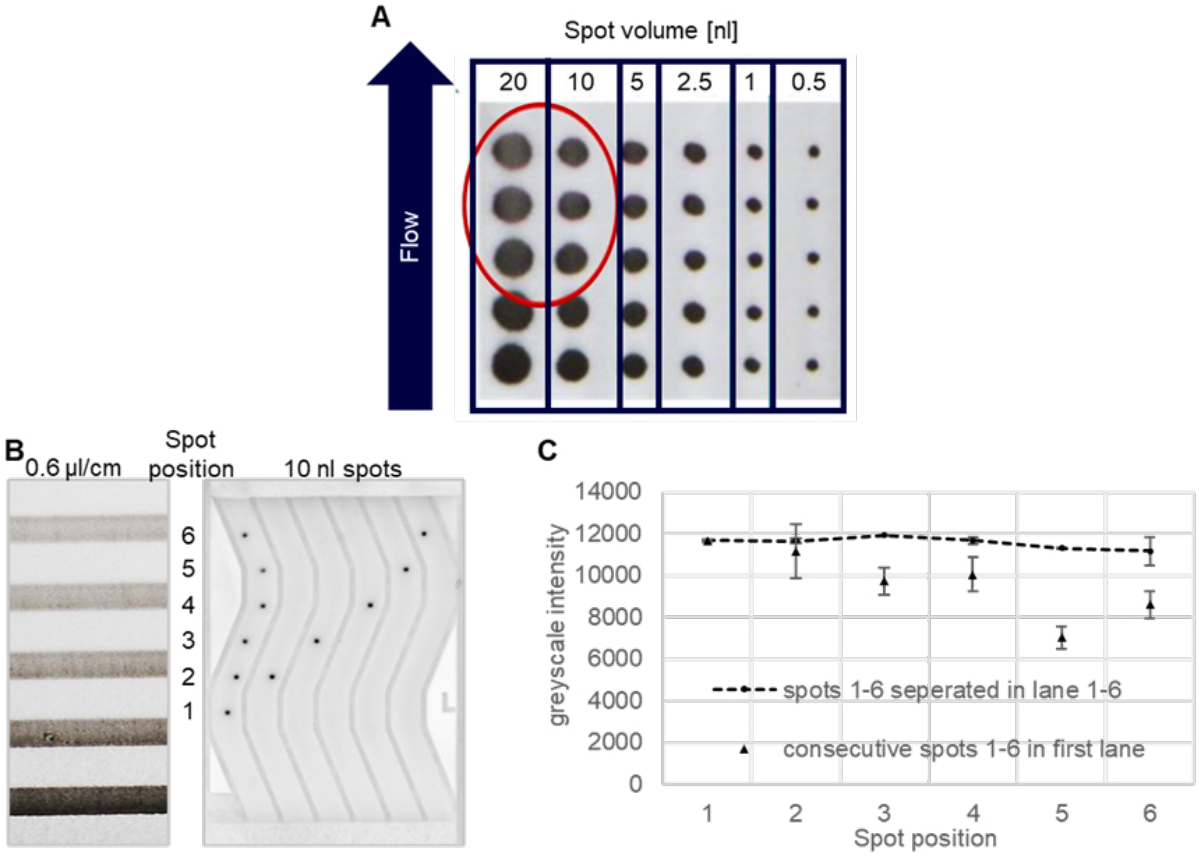

Figure 1:

Advantages of dot-based LFA: A Comparison of a 5-fold multiplex LFA: due to the wind shadow effect, decreasing intensity for each dot (volume > 5 nl, red circle) in lateral flow direction is observable. B Comparison of a multiplex assay in line (left) and dot (right) format: decreasing intensity of lines and spots in lateral flow direction, which follow each other in a lane (consecutive spots), while one spot per lane shows constant intensity C Quantitative readout of B using Epson Perfection V600. Greyscale intensity describes the pixel value of a 8-bit image analyzed in ImageJ, the absolute value is determined from the area under the peak (AuP) in distinction to the background.

Advantages of the Unisart StructSure® Membrane

The flow dynamic of a sample fluid plays an essential role in the effectiveness of an LFA, influencing the transport and interaction of analytes and antibodies within the system. The Unisart StructSure® membrane provides a specific flow pattern directly affecting the performance of an LFA. Capillary forces show an inverse relationship to the channel width according to the Young-Laplace equation [Olanrewaju, et al. (2018)]. As driving force in LFA this increase in narrower channels enhances the sample flow,
requiring less sample volume to achieve the same flow distance. This can be advantageous when dealing with limited sample volumes, making the assay more suitable for applications with scarce samples. Furthermore, the greater force exerted causes an increase in the flow velocity resulting in an earlier arrival of the analyte-detector mix at the capture antibody. The direct comparison of the Unisart StructSure® membrane with the regular non-structured Unisart® membrane of the same type (CN140) and stick width showed a 48 % and 35 % faster time to pass the membrane with water and serum respectively, providing an improved fluidic flow of viscous solutions.

Furthermore, reducing the channel width from 14 mm to 6 channels of 2 mm each as shown in the Unisart StructSure® membranes promotes laminar flow since the Reynolds number (Re) a, as parameter to predict the flow regime decreases with the characteristic length (channel width in this case) indicating a laminar flow for low Re.

This type of fluidic flow is characterized by smooth and organized layers of fluid moving in parallel providing more predictable and reproducible flow profiles, which is crucial for designing precise and controlled experiments. Laminar flow reduces dispersion, allowing for more localized and controlled interactions of the assay components. [Kenis, et al. (2000)]
Also, controlled mixing of the sample with the assay reagents promotes more efficient interaction and thus an increased sensitivity. To achieve this mixing, turbulences are generated by the curved shape as different parts of the sample experience increased pressure near the outer area of the channel within the curve and therefore varying velocity, leading to dispersion of the analyte (Figure 2) [Krzyk, et al. (2018)]. The enhanced mass transfer within the fluid brings the analyte into contact with the detector and capture antibody for improved binding. The combination of laminar flow and controlled generation of turbulence results in beneficial fluid dynamic, while excessive dispersion could affect the assay accuracy.
Another characteristic of the wave-like structure is the extension of the distance to the absorbent pad from 25 mm of the regular membrane to 26.2 mm at the Unisart StructSure® membrane, allowing a greater distance for the capture molecule and thus a longer incubation time between analyte and detector. Depending on the assay requirements this extension can provide a more flexible assay layout.

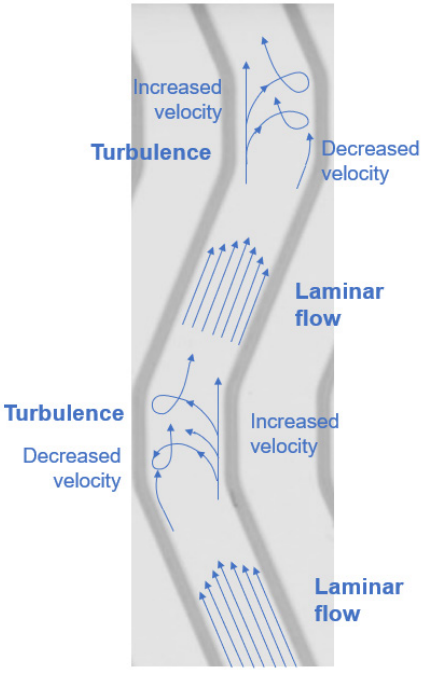

Figure 2:

Flow dynamics of Unisart StructSure® membranes
Narrow channels provide laminar flow in straight sections while turbulences occur during curvature due to velocity changes. The faster flow at the inner wall compared to reduced velocity at the outer wall results in the overlapping of fluids and swirls.

Case Study - Sepsis Multiplex LFA

Sepsis is a life-threatening condition that occurs when the body's response to infection becomes dysregulated, leading to widespread inflammation and organ dysfunction. Early and accurate diagnosis is crucial for timely intervention and improved patient outcomes. The most popular biomarkers for diagnosis of Sepsis are Procalcitonin (PCT), which typically remains at low blood concentrations but rises significantly in the presence of bacterial infections, especially sepsis, and C-reactive protein (CRP) as a general inflammation marker. Since CRP is a sensitive marker but not specific for sepsis the combination with PCT as a more specific but less sensitive marker provides a balance, improving both sensitivity and specificity in sepsis diagnosis.

In addition, PCT ensures early detection of sepsis and distinguishes between bacterial and non-bacterial inflammation leading to more accurate and targeted treatment decisions. Furthermore, measuring the concentration of both PCT and CRP provides information about the severity of sepsis supporting acute patient management and monitoring.

Leveraging their complementary strengths, the method presented in this white paper is used to establish a multiplex LFA combining PCT and CRP in three concentrations each as a cut-off level for different severity stages of sepsis (Table 1). By assessing the presence/absence of the spots and corresponding cut-off values, a semi-quantitative evaluation by eye is possible but can be extended to a quantitative evaluation by using the sciREADER LF2 (SCIENION GmbH, Outlook) with integrated kinetic measurement algorithms.

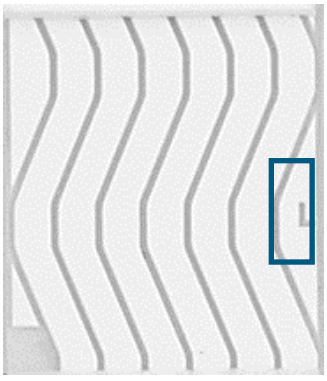

Figure 3:

Target specific fiducial as reference point for precise spotting with correct target position and orientation.

Biomarker	Indication	Cut-Off
Procalcitonin (PCT)	Possible systemic bacterial infection	≥ 0.5 ng/ml
	Likely systemic bacterial infection	≥ 2 < 10 ng/ml
	Highly likely severe bacterial sepsis or septic shock	≥ 10 ng/ml
C-reactive protein (CRP)	Likely mild inflammation or viral infection	≥ 10,000 ng/ml
	Likely active inflammation or systemic infection	≥ 40,000 - 200,000 ng/ml
	Severe infection or injury	≥ 200,000 ng/ml

Table 1:

Biomarker for detection of sepsis with blood concentrations as cut-off level indicating different stages of severity in the course of sepsis.

Materials and Methods

Multiplex arrays were printed with SCIENION's sciFLEXARRAYER SX (Figure 4) precision microdispenser on Unisart CN 140 StructSure® membranes (Sartorius, 3UN14ER066S01WS).
In brief, 10 nl of three different concentrations of anti-PCT and anti-CRP antibodies (HyTest) were deposited as described in the assay layout in Figure 5. Second anti-PCT and anti-CRP antibodies were conjugated with carbon nanoparticles to detect the analytes, PCT and CRP, in a sandwich assay format. As a result of the dual high-resolution cameras (Figure 4, Drop and Head cameras), the sciFLEXARRAYER system provides full control over drop generation and target positioning, allowing to verify the quality of the printout.
With the drop camera, the generation of droplets, as well as the drop volume, quality, and deviation from the printing axis were monitored, revealing stable droplets at the nanoliter scale (Figure 4,5). At the end of the printing process, the head camera allowed inspection of the target and allows to use only membranes with spots that fulfill specific quality criteria.
The mastercard was assembled using a backing card (Kenosha), a printed membrane, an absorbent pad (Whatman CF5, Cytiva) and a sample application pad (glass wool fibers, Kenosha). Afterwards, the card was cut (Matrix 2360 Shear Cutter, Kinematic Automation) into 14 mm strips ensuring the integrity of each membrane structure. Test-run was performed by putting the sticks into a perfect-fitting cassette developed in cooperation between Sartorius and SCIENION GmbH and applying a mixture of conjugate and sample. After an incubation time of 30 min, sticks were measured and analyzed using the sciREADER CL2 with integrated analyzing software (SCIENION GmbH).

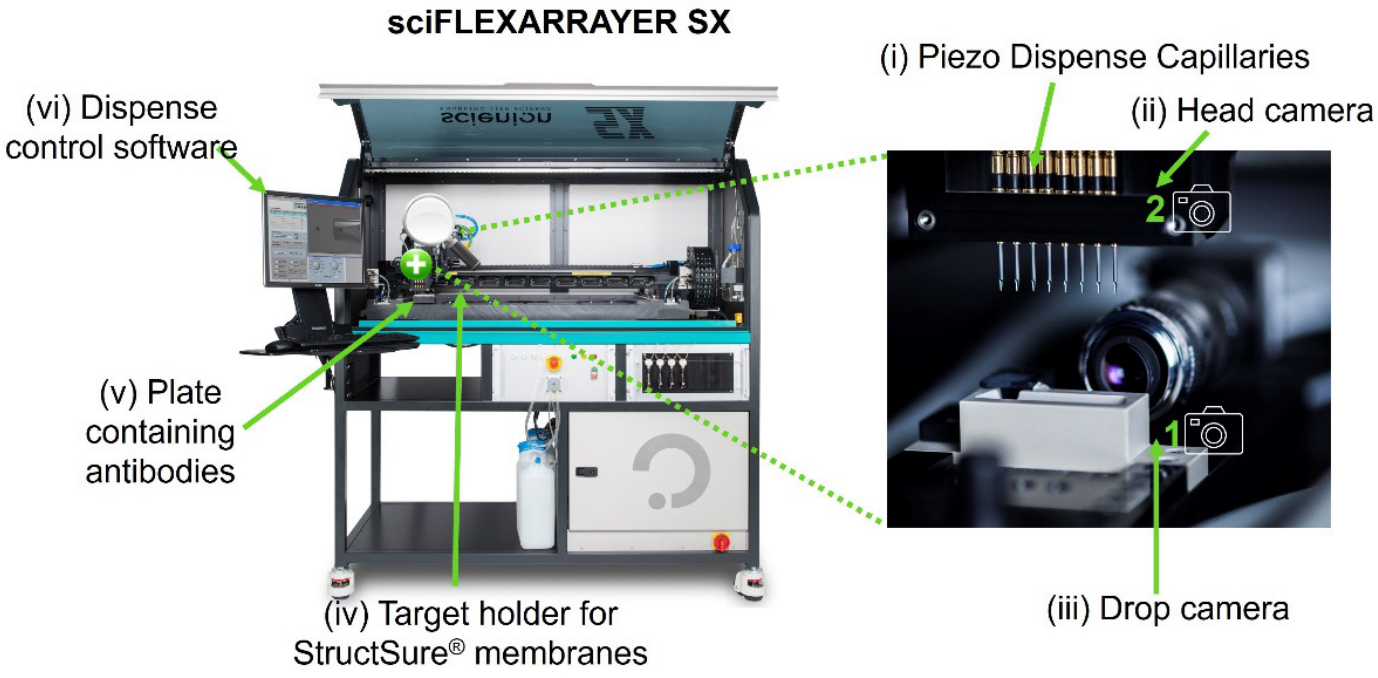

Figure 4.

Overview of the sciFLEXARRAYER S11 (SCIENION) precision microdispenser for depositing of antibodies on Unisart CN 140 StructSure® membranes. Arrows and numbers indicate the presence of (i) the Piezo Dispense Capillaries; (ii) the head camera used for imaging membranes after printing; (iii) the drop high resolution camera allowing to visualize and quantify drop parameters; (iv) the target holder for the membranes; (v) and the location of a 96-well plate containing antibodies to be printed (vi) the computer screen with the software controlling the sciFLEXARRAYER S11 microdispenser.

Results & Discussion (Proposed Solution)

Structured membranes in combination with precision microdispensing show improved performance

Evaluation by Eye Possible

Dot-based multiplexing on an LFA is possible on any type of membrane. Due to the complexity of the assay, evaluating the pattern by eye may be challenging, so analysis via a reader is recommended. Due to the structural design of the Sartorius Unisart StructSure® membranes, visual spots can be assigned to corresponding biomarkers, simplifying evaluation without a reader. In the case of the established Sepsis Multiplex assay, it is easy to differentiate between the three spots for CRP in the three left channels of the membrane structure and those for PCT on the right even if not all spots occur (Figure 5) ensuring a semiquantitative analysis and decision about the severity and type of infection (bacterial, non-bacterial). The small channels allow better control over the positioning of different capture molecules within the detection area. However, due to the size of the spots, the decision of the presence or absence of a spot remains subjective and therefore it is meaningful to use a reader. This assignable positioning requires high precision spot dispensing, which is accomplished by SCIENION's sciDROP PICO dispensing technology. Contactless printing assures the integrity of the membrane resulting in homogenous spots with controlled drop volumes for optimal assay performance.

Unisart StructSure® Multiplex

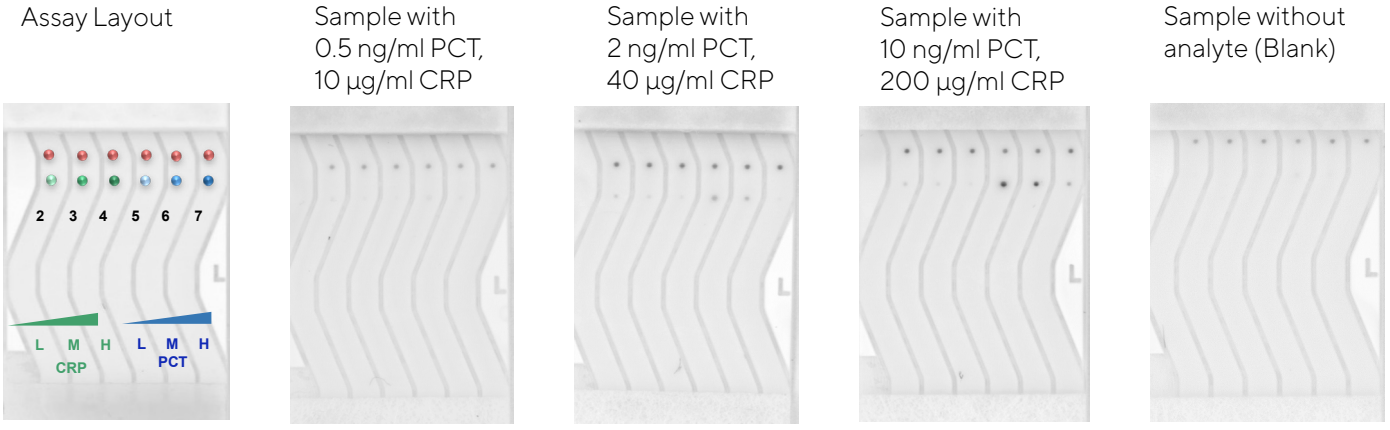

Figure 5:

Sepsis Multiplex assay on Unisart StructSure® membrane: Semiquantitative evaluation by presence/ absence of the respective spots in the cut-off area. 1: a-mouse IgG, assay control; 2: Cut-off for 10 µg/ml CRP (L); 3: Cut-off for 40 µg/ml CRP (M), 4: Cut-off for 200 µg/ml CRP (H); 5: Cut-off for 0.5 ng/ml PCT (L); 6: Cut-off for 2 ng/ml PCT (M); 7: Cut-off for 10 ng/ml PCT (H)

Increased Signal Intensity

As described before the structured membrane provides beneficial flow dynamics and increased incubation time between the analyte and antibodies due to an enlarged reaction area. In comparison to the same batch of unstructured Unisart® CN140 membrane, the structured membrane achieves an increase in signal intensity of 52 % in dependence on spot position (Figure 6).

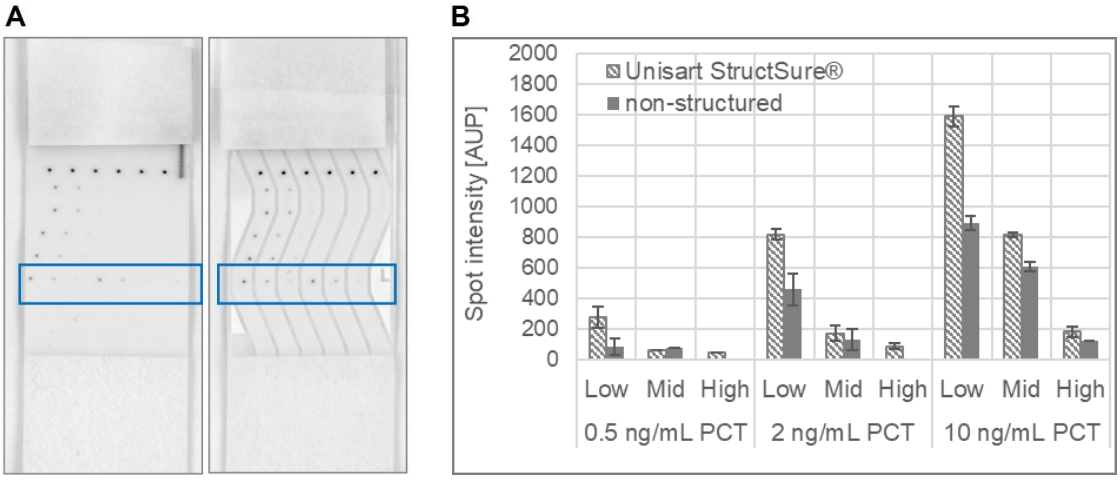

Figure 6:

Comparison of Unisart StructSure® with non-structured membrane. A Semiquantitative evaluation of spots with 10 ng/ml PCT sample on non-structured membrane (left panel) and Unisart StructSure® membrane (right panel). Spots within blue rectangular are analyzed as duplicate for B Comparison of spot intensities of 0.5, 2, 10 ng/ml PCT on Unisart StructSure® and non-structured membrane

Specific Cassette Improves Assay Reproducibility and Time

The properties of fluid dynamics are supported using a cassette designed for the Unisart StructSure® stick dimension (Figure 7 A). By ensuring even transition between materials, the cassette ensures a controlled and homogeneous flow throughout all channels, thus ensuring higher reproducibility. This is especially shown for incubation times when signal intensity is not yet fully developed resulting in 10 % less CV after 30 min incubation of the Sepsis multiplex LFA using the cassette instead of loose sticks (Figure 7 B). Therefore, this point-of-care test for everyday laboratory or home use can be conducted in a shorter amount of time rather than waiting for complete signal development (hereafter 60 min).

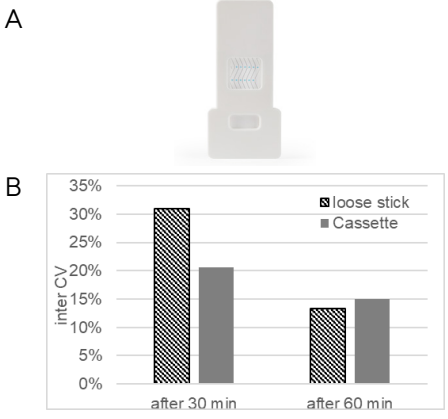

Figure 7:

Improvement of fluid dynamics using the LFA cassette. (A) Picture of cassette for 14 mm Unisart StructSure® and regular Unisart® LFA sticks, designed and created in collaboration between SCIENION GmbH, and Sartorius (B) Comparison of the coefficient of variation (CV) of control spot signals after 30- and 60-min incubation with and without cassette. 16 sticks including 6 control spots each per variant were tested.

Simultaneous Detection of Analytes With Very Different Abundance

In addition to the well-known quality of Sartorius Unisart® membranes, the properties of the Unisart StructSure® membrane permit simultaneous detection of analytes with very different abundances of 4-5 logs differences in concentration on one stick. When combining biomarkers with large abundance differences in the required measurement range it can be challenging to improve the sensitivity of one biomarker while lowering the sensitivity of another since most assay components like sample buffer, sample volume, sample dilution, solid phase materials, and fluid dynamics are directly affecting both assays. By adding an unlabeled anti-CRP antibody, which competes with the conjugate for binding with the overabundant CRP analyte, the sensitivity of the CRP assay could be significantly reduced without compromising the required sensitivity of the PCT assay. By varying the respective capture antibody concentrations, it was possible to distinguish between PCT concentrations in the ng/ml range while detecting CRP in µg/ml concentrations (Figure 8).

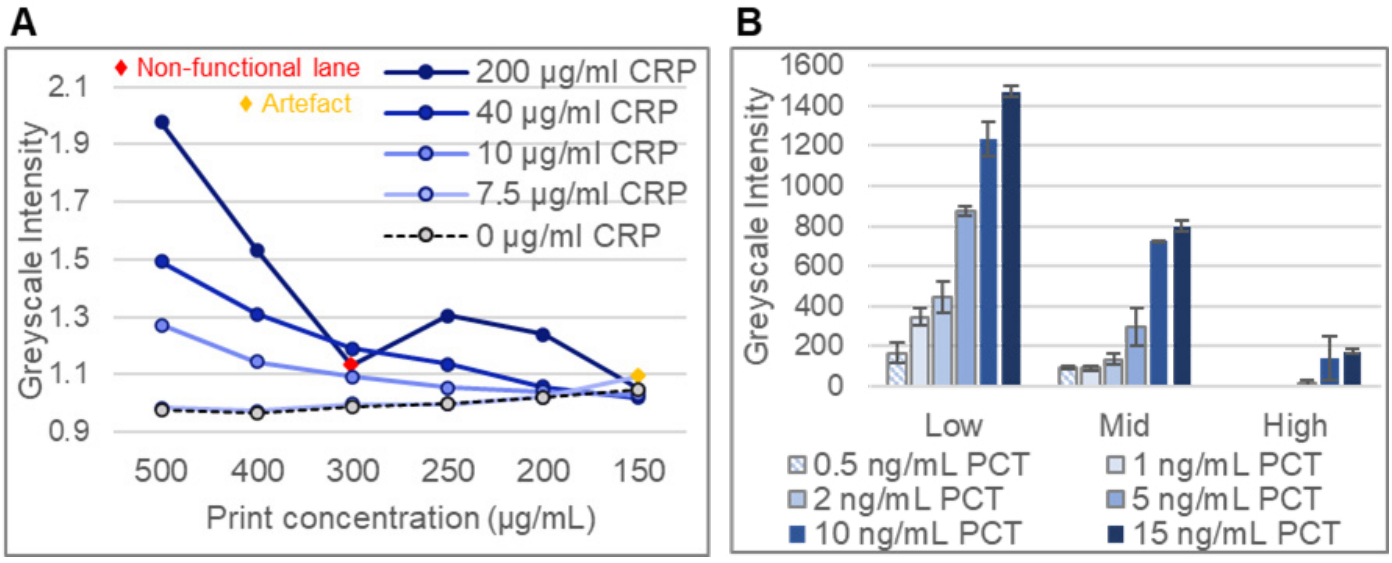

Figure 8:

Varying capture antibody concentration to reach required sensitivity **A** Titration of CRP analyte concentration in dependance of the anti-CRP capture antibody concentration and PCT analyte concentration at cut-off spots Low, Mid, High **B** Differentiation of CRP and PCT cut-offs is possible at varying capture antibody values.

Outlook & Future Direction

Quantification With SCIENION´s Reader Technology

Semiquantitative evaluation of CRP and PCT cut-off values was possible using the Unisart StructSure® membrane but differentiation between the presence and absence of spots at the cut-off values would be much easier using SCIENION's reader technology. The sciREADER LF2 is a portable, tiny device designed for kinetic and end-point analysis of LFA, especially for the analysis of spotted arrays consisting of multiple detector regions ensured by a powerful grid alignment and spot-finding algorithm.

Figure 9:

sciREADER LF2 for quantitative readout of LFA assays

Sandwich-immunoassays for the detection of highly expressed blood markers like CRP for sepsis, are often limited by the hook effect. By measuring the signal intensities of test and control spots in real time the change of the test-to-control ratio (T/C) is observable indicating the varying signal development rate for low and very high analyte concentrations while the final T/C value may be the same at endpoint measurement [Rey, et al. (2017)]. This makes it possible to extend the dynamic range of a LFA through kinetic measurements. Furthermore, kinetics enables the identification of reactions at early stages, allowing to stop a measurement as soon as spots occur in case of cut-off value detection reducing the assay time. Since the development rate of the signal intensity is proportional or anti-proportional to the analyte concentration in a sandwich or competitive assay, respectively, quantification can be done using the slope between different measuring points and referring it to a pre-characterized standard curve, which can be defined in the reader program as a reference. This enables quantification without carrying separate standards per test run.

Increase Specificity by One Conjugate per Lane

In fullstick format the conjugate is usually dispersed and dried onto a pad suitable for resolubilization by the sample. There are various approaches to integrate the detector antibody on a multiplex assay like simultaneous conjugation, mixing of individually conjugated antibodies fixing all detectors at once on the conjugate release pad for both options or spatial separation of single conjugates on the pad. Anyway, the detector antibodies get mixed up during the run as the sample-conjugate-mixture flows across the membrane together entailing the risk of cross-contamination and unspecificities. The subdivision of the membrane at Unisart StructSure® now offers the new possibility of total separation of the respective detectors avoiding the contact between antibodies not crucial for the reaction and therefore increasing the specificity. Initial experiments have shown promising results when applying the conjugate directly into one lane of the structured membrane after it has been pre-blocked to avoid binding between membrane and conjugate. Furthermore, the separated lanes provide the potential of placing hydrophobic barriers in some lanes achieving different reaction times to support the respective biomarker-antibody-kinetics and therefore to meet the requirements for the detection of biomarkers of different concentration ranges. In perspective, studies are being conducted on the ability of the conjugate to be resolubilized from the membrane and the associated assay functionality as well as improving the simultaneous detection of biomarkers with different abundances by hydrophobic treatment of the membrane.

Acknowledgments

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