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High Sensitivity Assay Development on the LSA®

Introduction

While SPR assays have the advantage of being able to detect binding interactions in a label free manner, this does not preclude the use of signal enhancing label strategies.

The lower quantitation limit of surface plasmon resonance (SPR) assays is typically considered to be limited by the mass of the observed analyte and the affinity of the capture system. This limit is typically orders of magnitude less than classic immunological assays using labeled detection systems. When signal enhancement strategies are applied, SPR can achieve lower limit of detection (LOD) levels comparable to or more sensitive than conventional labelled techniques such as Enzyme-Linked Immunosorbent Assay (ELISA).[1,2,3] In this example, a commercially available ELISA reagent kit for the measurement of human IL-6 was adapted for use on the Carterra® LSA® instrument. The assay performance exceeded the manufacturer's reported ELISA sensitivity limit and the LSA allows for many of the assay steps to be automated. This assay utilized a horseradish peroxidase streptavidin conjugate (SA-HRP) and a precipitating substrate mix, 4-chloro-1-naphthol and 3,3'-diaminobenzidine tetrahydrochloride (CN/DAB) for detection. Other signal enhancement strategies are also possible, some which would even allow for regeneration to even further increase the automated throughput possible on the platform.

Key Takeaways

- Enhancing the lower limit of detection (LOD) in the LSA extends the range of assay options available.
- Described here is an approach to enhancing LOD for a cytokine using commercially available reagents.
- LOD was improved from mid-range ng/ml down to single digit pg/ml using this approach.
- The concepts described here are readily adaptable to alternative reagents depending on assay needs.

Method Details

To serve as a model system, the R&D Systems Human (hIL-6) Duoset ELISA reagent kit (DY206-05) was used for the basis of the testing. Two tests were run covering different ranges of hIL-6 dilutions. The first test started with a much higher concentration (55 ng/mL) and was appropriate for label free and non-enzymatic detection of the hIL-6, but was far too high for the enzymatic signal amplification to remain in range. The second experiment utilized concentrations more appropriate for the enzymatic amplification and ranged from 2 ng/mL to 0.9 pg/mL. **Figure 1** highlights the overall workflow.

Capture surface preparation: A CMD200M sensor chip (Carterra, p/n 4280) was immobilized with a high-density capture lawn using the single flow cell (SFC) of the LSA. To prepare the lawn, the LSA was primed into 1x HBSTE buffer (Carterra p/n 3728) and the sensor chip was preconditioned with a 1 minute pulse of each of three solutions: 50 mM NaOH (Carterra p/n 3638), 1M NaCl, and 10 mM Glycine pH 2.0 (Carterra p/n 3640). After preconditioning, the chip was activated with a 10 minute injection of a freshly prepared 1:1:1 (v/v/v) mixture of 0.4MEDC + 0.1MN-hydroxysulfosuccinimide (S-NHS) + 0.1M 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.5 Carterra (p/n 3625). The anti-human IL-6 capture antibody from the kit was buffer exchanged with

Figure 1. Enhanced Sensitivity Workflow

a spin desalting column into 10 mM Sodium Acetate pH 5.0 (Carterra p/n 3805) with 0.05% Tween-20 (Carterra p/n 3621) and then further diluted to a final pH of approximately pH 4.75 using 10 mM NaAcetate pH 4.5 (Carterra p/n 3802). The antibody was injected for 15 minutes at approximately 70 µg/mL and immobilized between 6000 and 7000 RU on the array surface. The surface was then quenched with a 7-minute injection of 1 M ethanolamine pH 8.5 (Carterra p/n 3626).

Human IL-6 capture and detection: For the IL-6 capture and detection steps, the LSA was primed with 1x HBSTE with 0.5 mg/mL BSA. This running buffer was also used as the diluent for the IL-6 samples, the detection antibody, and the SA-HRP conjugate.

Titrations of the hIL-6 standard in the kit were prepared in either running buffer (RB, designated in blue-colored font) alone or 10% normal human serum (Designated in red-colored font, Jackson ImmunoResearch, 009-000-001). Human IL-6 dilutions were captured to the array under constant cycling using the 96-channel flow cell for one hour using the Capture Kinetics wizard. Samples were tested in quadruplicate for each condition, with 8 concentrations of hIL-6 with and without the 10% normal human serum **(Figure 2.A)**. In addition, titrations of normal human serum without the spiked hIL-6 were tested starting at 3% to check for background IL-6 in the normal serum.

The biotinylated anti-human IL-6 detection antibody was injected at 5 µg/mL for one hour **(Figure 2.B)**. This was then followed by an injection of the SA-HRP at a 5x dilution from the ELISA Kit for 30 minutes **(Figure 2.C)**.

The CN/DAB (ThermoFisher #34000) was injected as a 15x dilution in the supplied dilution buffer from the 10x stock for 7 minutes with a 5 minute dissociation **(Figure 2.A)**.

Date analysis: These data were processed in Carterra's Kinetics™ analysis software tool. Report point data from the final CN/DAB step was exported and processed in GraphPad Prism 9 and presented with a five-parameter logistic fit. This five-parameter logistic is the same model

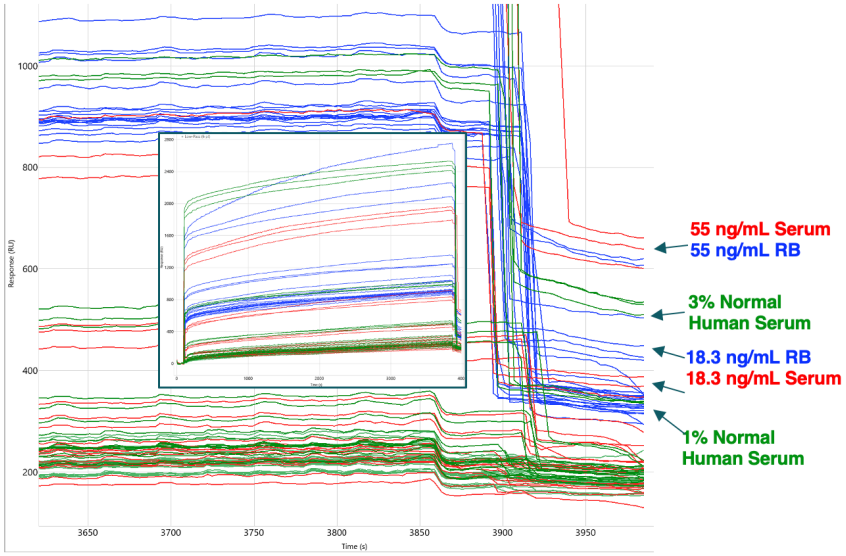

Figure 2A. Direct detection of hIL-6

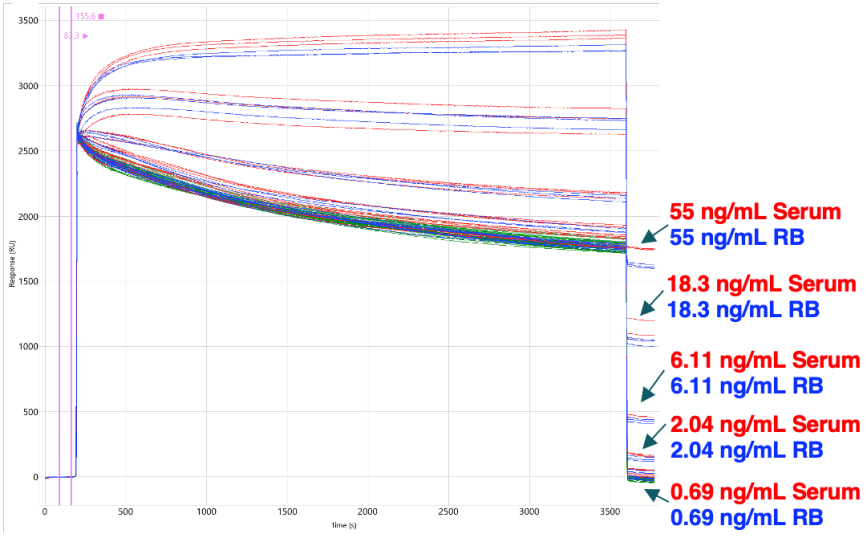

Figure 2B. Injection of anti-hIL-6-biotin detection mAb

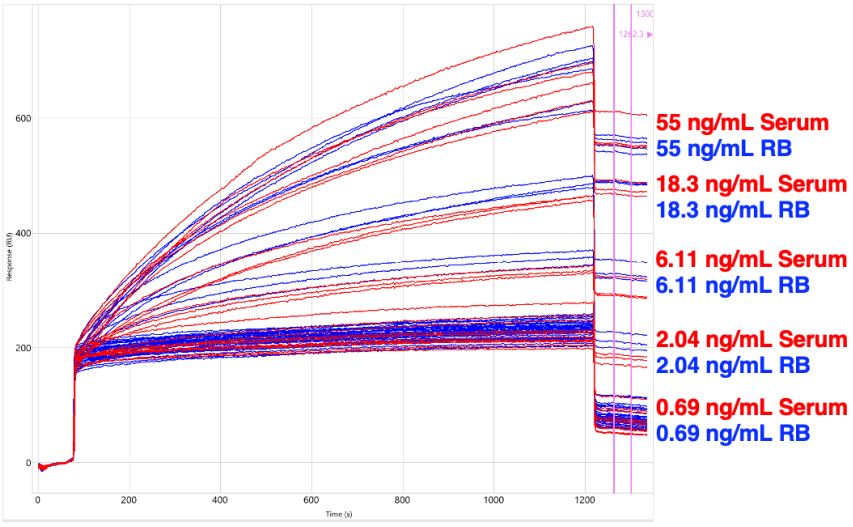

Figure 2C. Injection of SA-HRP

the Kinetics software uses to extrapolate concentrations for unknowns.

Results and Discussion

This experiment demonstrated a number of concepts on the LSA platform. First, that it is very straightforward to adapt existing immunoassay formats, such as ELISA reagents, to the LSA. Secondly, the automated sample handling and rigorous washing of samples in the flow cell provides a very low background and highly automatable mechanism to analyze high sensitivity immunoassays with enzymatic detection.

In this example the capture lawn was prepared as a separate run. The next three steps were fully automated using queued assay wizards. The Capture Kinetics wizard was used to capture the hIL-6 samples and then inject the biotinylated anti-hIL-6 detection mAb. Next a queued Quickstart assay was used to inject the SA_HRP solution. Lastly, another Quickstart wizard was used to inject the CN/DAB solution. It is likely that the CN/DAB injection could have also been queued into the same run with the other samples but the stability of the diluted CN/DAB for the 2.5 hour capture and detection steps had not yet been determined. If an appropriate capture surface was prepared ahead of time or manufactured for the assay's purpose, the entire experiment could be automated for up to 384 samples with a simple set up of the sample plates and three small vials containing 270 mL of the detection antibody, the SA-HRP, and the enzyme substrate.

The first assay showed that using non-enzymatic SPR detection can offer significant enhancement of signals. In direct observation of hIL-6, binding the sensitivity was approximately 50 ng/mL **(Figure 2.A)**. Some small background binding of serum proteins was also present which would prevent the visualization of any lower concentrations of the non-enhanced signal. When the biotinylated detection antibody was injected, the background from the serum was no longer evident and the nearly 7.5 fold larger mass of the antibody (150 kDa IgG versus 20.3 kDA for human IL-6) allowed additional sensitivity with a clear detection of IL-6 down to 2 ng/mL **(Figure 2.B)**. The ability to discern direct signal from the binding of the SA-HRP was generally equivalent to that of the detection antibody, showing a 2 ng/mL sensitivity, as well **(Figure 2.C)**. It is quite possible that if the biotinylated antibody had been premixed with a slight excess of SA-HRP, the binding could have been visualized in a single step with an even larger mass increase and small sensitivity gain. One can envision the delivery of an even larger detection molecule or one with a high refractive index which would further enhance the non-enzymatic detection signal.

The interest in evaluating non-enzymatic detection is generally due to the irreversible nature of the precipitating substrate. If labelled affinity-based detection reagents are utilized, they can likely be regenerated from the surface to further expand the unattended throughput of the assay. With the CN/DAB solutions, the enzymatic product is an insoluble mineral which attaches to the chip surface. This enzyme conversion is extremely efficient and only very small quantities of bound SA-HRP are required to provide large, amplified signals. In the high concentration test, most the samples were out of range of linear detection rapidly and were not useful for numerical quantitation and evaluation. The 2 ng/mL concentration of hIL-6 appeared to be at the very top of the effective dynamic range of the system at over 17000 RU and some ROIs going in and out of detectable limits. The signal for the 667 pg/mL sample was high but clearly in the detectable range throughout the injection **(Figure 3)**. On the lower end of the detection limit **(Figure 3. right inset panel)**, the 2.7 pg/mL sample was the lowest concentration which was non-overlapping with negative controls and serum alone samples, demonstrating single digit pg/mL sensitivity **(Figure 4)**. The concentration range appropriate for analysis using enzymatic detection appears to start where the mass-based SPR signal alone ends and the binding sensorgrams of the second lower concentration experiment showed little evidence of activity beyond 2 ng/mL until the enhancement solution was delivered.

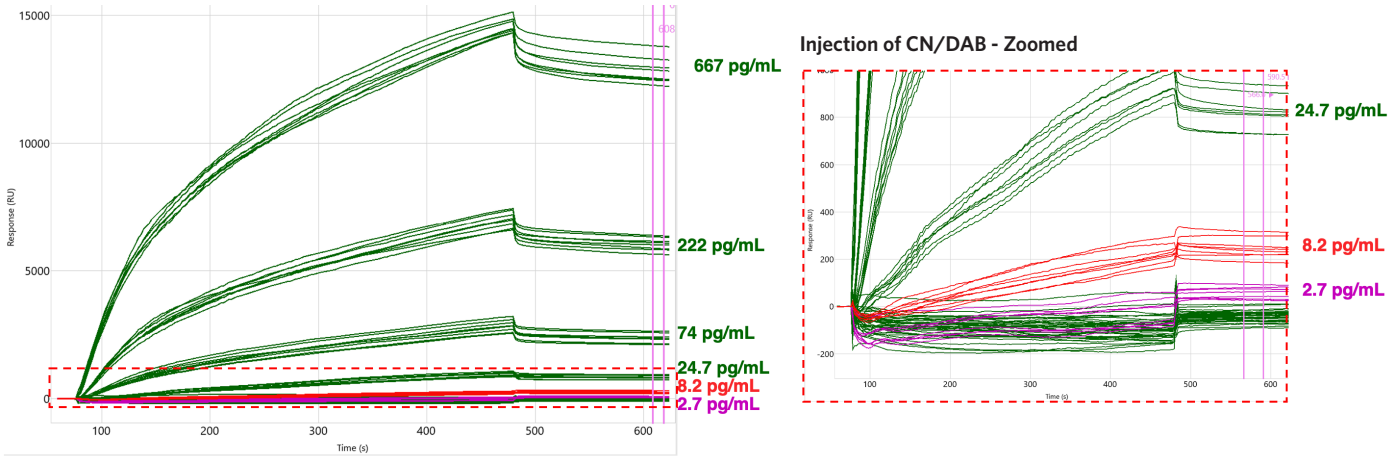

Figure 3. Sensorgram data for the injection of CN/DAB showing HRP driven formation of precipitating substrate. The 8.2 pg/mL is colored red and the 2.7 pg/mL purple for better visualization. The pink vertical bars represent the region selected for creation of a report point for further analysis.

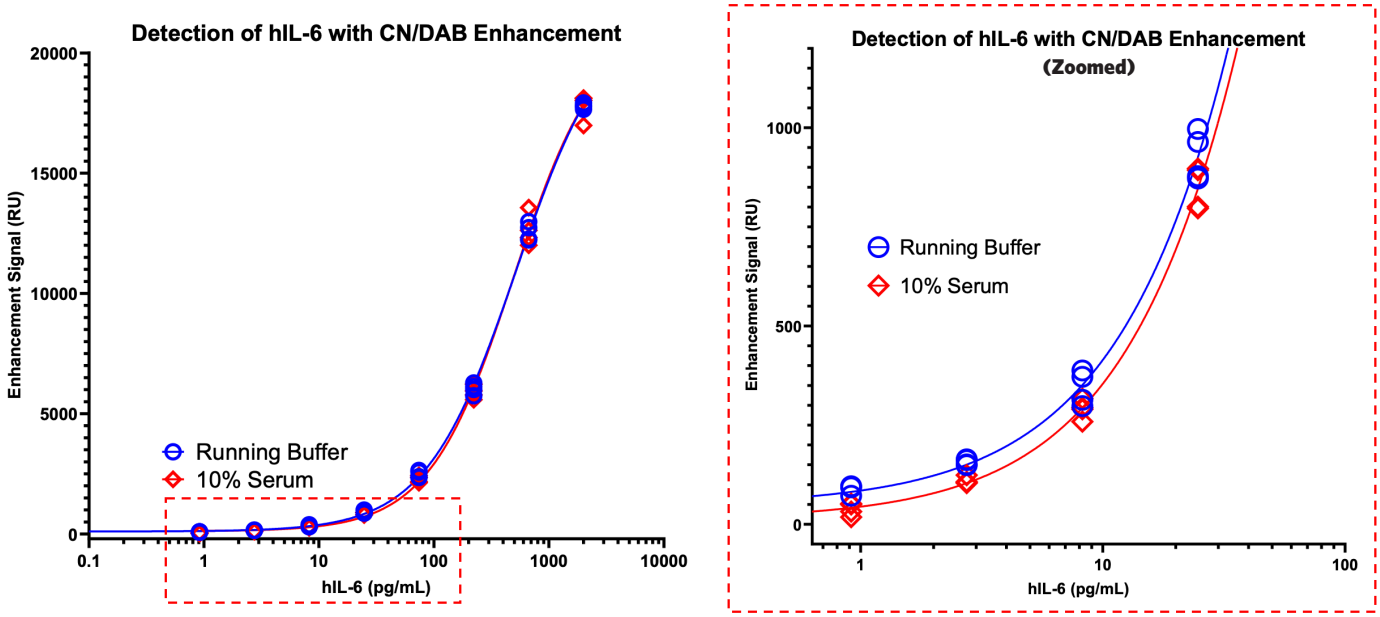

Figure 4. The enhancement signal versus hIL-6 concentration is shown for the running buffer dilutions (blue circles) and in 10% serum (red diamonds). The panel on the right encased in the red box represents a zoomed-in view of the data show in the left panel to highlight the results.

Summary

These tests demonstrate the capability of the LSA to perform an automated detection of a cytokine in diluted human serum in a highly parallel manner and with a sensitivity as good or better than a standard ELISA. With proper optimization and range finding, this assay strategy could be used to measure up to 384 samples in a fully automated run in about five hours. These data also clearly demonstrate the ability of signal enhancers to improve both the detection limit and specificity of SPR detection.

References

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