Identification of Spurious Signal in Immunoassays Due to Serum Antibodies Against Blocking Proteins

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Abstract

Background: Many immunoassays are currently performed using various forms of bovine casein to block the non-specific binding of sample constituents and other assay reagents to the underlying substrate. However, the possible presence of immunoglobulins against casein in test samples is often not considered during assay development and can lead to erroneous results.

Methods: A serological assay was developed for the detection of serum antibodies against the intestinal nematode *Strongyloides stercoralis* using a novel diffractive optics-based biosensor platform. This technology enables the label-free detection of molecular interactions in real- time and thus facilitates the monitoring of each reagent incubation step during assay development. The steps in this assay consisted of: 1) blocking using a commercially available casein-based blocking solution; 2) immobilization of a *Strongyloides* recombinant antigen on the sensors; 3) incubation of patient serum diluted 20-fold in a commercially available serum diluent designed to eliminate low- and medium-affinity interactions; and 4) amplification of antibody binding using goat anti-human IgG coated gold nanoparticles.

Results: An analysis of over one hundred patients sera by this method led to the identification of a subset of samples, representing 7% of the total, that displayed inexplicable reactivity to the assay surface. This reactivity was determined to be caused by the presence of anti-bovine casein antibodies in the subset population which bound to the casein used to block non-specific binding to the sensors. The addition of free casein to the serum diluent successfully eradicated this unexpected reactivity.

Conclusions: The use of a label-free, real-time biosensor platform enabled the detection of background reactivity of serum antibodies against bovine casein found in a subset of patients sera to contain casein antibodies, thus reacting with the casein used to block the assay substrate. In standard plate-based enzyme-linked immunosorbent assays using a casein-based blocking method, samples containing antibodies to bovine casein may lead to artificially elevated signal and false positive results. These results highlight the potential problems posed by the presence of antibodies against blocking proteins in patient sera and the importance of developing strategies to eliminate their reactivity when developing immunoassays.

Introduction

Bovine serum albumin (BSA) or casein are commonly used to block non-specific binding of assay constituents to the underlying substrate. However, when performing serological assays, the presence of antibodies against BSA or bovine casein in the patient sera can lead to false positive results when general species specific antibodies are used for detection. The presence of IgG antibodies to bovine proteins in human sera is well documented in the literature and is believed to arise from immune responses to ingested bovine products such as milk or from bovine proteins included in drug formulations. It is therefore important to consider the possible presence of these antibodies in patient samples when developing immunoassays. Here, we report the inadvertent detection of bovine casein in a subset of human sera that were tested using a novel diffractive optics technology while developing a serological assay for *Strongyloides stercoralis*. Due to this unique detection system, these samples displayed a characteristic negative signal which indicated aberrant binding. The discovery, characterization and resolution of this binding are described.

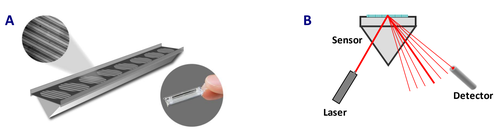

Figure 1: (A) The dotLab Sensor; (B) Cross-sectional view of diffraction image generated by the illumination of sensor spots on a sensors with a laser beam.

Principles of Diffractive Optics Technology

Diffractive optics technology (dot®) utilizes grating-based optical diffraction to provide a quantitative, label-free measurement of molecular interactions in real time. Core to the technology is the use of disposable plastic sensors each consisting of a contiguous array of interaction surfaces or spots lining the bottom of a 10 µL flow channel. The spots are composed of coupling reagent pre-patterned in a repetitive sequence of lines forming a diffraction grating (Figure 1A). Several attachment chemistries (eg: avidin, amine reactive, oligonucleotide-based addressing reagents for multiplexing) allow the user flexibility in coupling their own content to the sensors.

In the dotLab instrument, the sensor spots are illuminated with a low powered laser which generates a specific diffraction image (Figure 1B). The intensity of each diffractive order is proportional to the height of the lines that make up the grating (Figure 2). An optical prism integrated below the flow channel functions to reflect the diffraction image which prevents it from passing through the sample. This eliminates the effects of sample refractive index and color on signal intensity which allows for the direct analysis of crude biological samples without dilution.

Changes in diffraction image intensity caused by binding or dissociation events on the sensor surface are monitored in real time using a photodiode detector. The rate of change or amplitude of signal can be used to describe these events quantitatively.

A significant advantage to this approach is that it is inherently self referencing. Any species that binds non-specifically to both the lines and the space between the lines does not alter the relative height of the lines and thus has minimal impact on the signal. This property also allows for the use of vigorous mixing or changes in reagent flow during an assay without signal disruption. The ability to introduce samples to sensors in a mix mode permits extended binding experiments for low affinity interactions using small sample volumes, overcoming the consumption requirements of continuous flow systems.

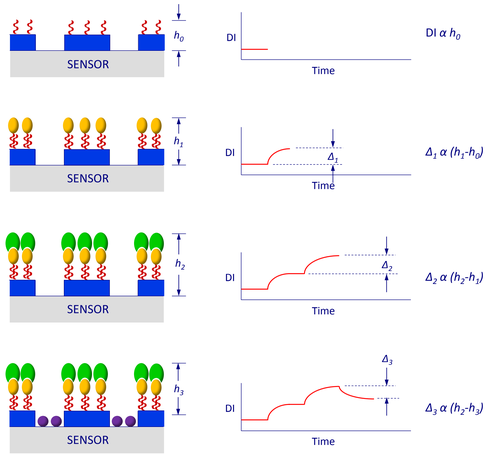

Figure 2: Principles of optical diffraction. The intensity of the diffraction image (DI) is directly related to the height of the lines making up the diffraction grating (h). As molecules bind to the lines, their height relative to the underlying substrate increases resulting in an increase in DI which is reported in real time by the dotLab mX. Molecular binding to the substrate leads to a reduction in the relative height of the lines and results in a decrease in DI.

Materials and Methods

Samples and Reagents:

Serum samples were obtained from: 1) parasitologically proven *Strongyloides stercoralis* infection; 2) other parasitic infection; 3) healthy individuals. NIE recombinant antigen from *S. stercoralis* L3-stage larvae was obtained from Dr. F.A. Neva (NIH). NIE conjugation to panelPlus D oligonucleotides was performed using the panelPlus Labeling Kit (Axela, Inc.). Goat anti-human IgG – gold nanoparticle (GNP) conjugates were obtained from Bioassay Works. panelPlus sensors and all other reagents were obtained from Axela, Inc.

dotLab-Based Serological Assay:

- all serological assays were performed using the dotLab® mX System and dotLab Serology Kit (Axela, Inc.)
- assays were conducted in a running buffer of HEPES-buffered saline (HBS) containing 0.1% v/v Tween-20 (HBST)
- sensors were washed briefly with HBST, then blocked with a commercially available blocking buffer made up of hydrolyzed casein
- sensors were then incubated with NIE conjugated to panelPlus D oligonucleotide (NIE@D) resulting in the immobilization of NIE@D to the spots on the sensor
- serum samples were diluted 1:20 in either a commercial casein-free serum diluent designed to minimize mid- and low affinity interactions or commercial serum diluent containing 5% v/v hydrolyzed casein-based blocking buffer, and then incubated on the sensor
- serum NIE antibody binding signal was amplified using a 1:10 dilution of a goat anti-human IgG – GNP conjugate

Results

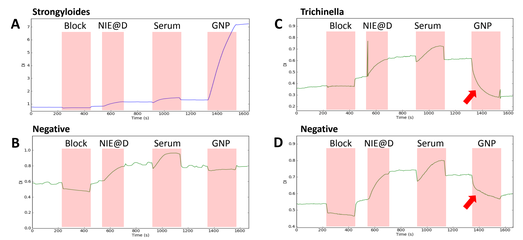

Figure 3: Representative dotLab traces of: (A) Strongyloides positive serum; (B) negative serum; and (C+D) sera demonstrating negative signal during GNP incubation. Of 115 sera analyzed, eight (7%) displayed negative GNP signal.

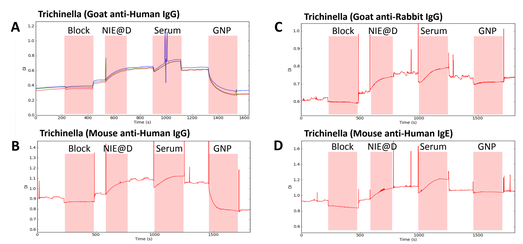

Figure 4: Negative GNP signal was observed specifically with GNP conjugated to anti-human IgG antibodies (A+B) and not with anti-rabbit IgG (C) or anti-human IgE (D) suggesting that human IgG might be binding between the lines.

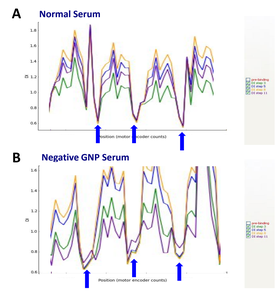

Figure 5: Surface scans of sensors following show the incremental increase of the height of the lines following each reagent incubation with no changes in height between the lines when analyzing normal serum (A). However, negative GNP serum displayed binding of goat anti-human IgG conjugated GNP in between the lines (B) indicating the binding of human IgG to the space between the lines. Given that the sensors were blocked with a casein-based solution, it was postulated that negative GNP sera contained anti-bovine casein antibodies which was binding in between the lines. If correct, the inclusion of free casein in the serum diluent should eradicate the negative GNP signal.

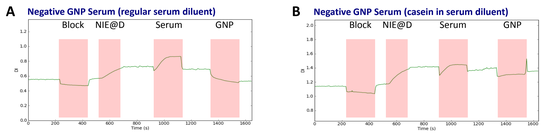

Figure 6: The inclusion of casein-based blocking solution in the serum diluent (5% v/v) successfully eliminated the negative GNP signal observed in a subset of serum samples.

Summary and Conclusions

- The results suggest that the inclusion of free casein in the serum diluent bound the serum anti-casein antibodies, restricting their availability to bind to casein immobilized to the sensors.
- When developing serological assays, the possible presence of antibodies against blocking reagents in the test sera needs to be considered.
- As demonstrated in this study, not all commercially available sets of blocking solution and serum diluent are formulated to address this undesired binding.
- Diffractive optics-based detection enabled the identification and characterization of serum samples containing antibodies against blocking proteins by generating negative binding curves. These samples would have generated false positive results on most immunoassay platforms.

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