Defence Research and Development Canada
Recherche et développement pour la défense Canada

Development and Characterization of a Suspension Bead Array Immunoassay for Ovalbumin

M. Snodgrass, T. Dickinson Laing, D.C. Mah, V. Lam, and C. Aw
Canada West Biosciences Inc

Contract Scientific Authorities:
H.S. Bhogal and R. E. Fulton
DRDC Suffield

Contract Report
DRDC Suffield CR 2006-248
December 2006

Canada

Development and Characterization of a Suspension Bead Array Immunoassay for Ovalbumin

M. Snodgrass, T. Dickinson Laing, D.C. Mah, V. Lam, and C. Aw
Canada West Biosciences Inc

Canada West Biosciences Inc
5429 60th Street
Camrose, AB
T4V 4G9

Contract Number: W7702-04-R044/001/EDM

Contract Scientific Authoritlies: H.S. Bhogal & R.E. Fulton (403-544-4507 & 544-4630)

Defence R&D Canada – Suffield

Contract Report

DRDC Suffield CR 2006-248

December 2006

This project was supported by the Defence R&D Canada Technology Investment Fund.

Abstract

The Suspension Array System (SAS) has enabled the development of assays that are rapid, sensitive and specific for any biological threat agent. In this study a SAS immunoassay was developed and characterized for the toxin simulant ovalbumin. Optimization of this assay and its performance are presented. Results obtained were compared with data obtained by ELISA. The limit of detection (LOD) for the SAS ovalbumin assay was 4.88 ng/mL, compared to a LOD of 9.5 pg/mL for the ovalbumin ELISA. When compared to the LODs for ovalbumin reported in a previous study, the difference between the assay sensitivities was considerably smaller, with an ELISA LOD of 300 pg/mL and a SAS assay LOD of 1 ng/mL. The 500-fold sensitivity difference was likely a reflection of the longer incubation times for the ELISA versus the SAS assays, where the incubation time for the ELISA was 1 hour compared to an incubation time of 30 minutes for the SAS. The trade off for this decrease in sensitivity is a rapid assay that can be performed in half the time as a traditional ELISA. The SAS ovalbumin assay had a well-to-well variance of 5.56%, while its plate-to-plate reproducibility was 14.2%. In comparison, the ELISA ovalbumin assay had a well-to-well variance of 4.90%, whereas its plate-to-plate reproducibility was 14.8%. In summary, an immunological assay that was rapid, sensitive and reproducible was created for a biological threat simulant by suspension array technology. The procedures used to develop the SAS assay for ovalbumin can be used as a template for development of other assays for toxins, bacteria, and viruses. SAS assays for individual biothreat agents can, in turn, be used as a starting point for the development of multiplexed assays for multiple agents.

Executive summary

Introduction:

In response to the threat of terrorist attacks using one or more of many toxins, bacteria or viruses, there is a need for the development of a rapid detection assay for multiple biological threat agents. The Suspension Array System from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, ON) utilizes Luminex bead technology to permit the multiplexing of up to 100 different assays for a single sample. A suspension array immunoassay was designed for ovalbumin, a commonly used protein toxin simulant, to evaluate the characteristics of this assay. The antigen sensitivity obtained by SAS was compared to that obtained by ELISA, the gold standard immunoassay for detection of analytes.

Results:

The limit of detection (LOD) for the SAS ovalbumin assay was 4.88 ng/mL, compared to a LOD of 9.5 pg/mL obtained by ovalbumin ELISA. The SAS ovalbumin assay had a well-to-well variance of 5.56%, while its plate-to-plate reproducibility was 14.2%. The observed 500-fold sensitivity difference was likely a reflection of the longer incubation times for the ELISA vs. the SAS assays; incubation time for the ELISA was one hour compared to an incubation time of 30 minutes in the SAS.

Significance:

The ability of the SAS assay to analyze multiple biological threat agents in a few hours may compensate for the loss of sensitivity compared to the ELISA, which takes multiple hours to complete. The procedures used to develop the SAS assay for ovalbumin can be used as a template for developing other SAS assays for toxins, bacteria and viruses. SAS assays for individual agents can be used for the development of multiplexed assays for multiple agents.

Future work:

Further SAS immunoassays will be developed for individual toxins, bacteria and viruses using the same procedures developed for the ovalbumin assay. These individual immunoassays will then be incorporated into multiplexed SAS immunoassays that can rapidly, and simultaneously, test for a number of biological threat agents. This format, when fully developed, may thus be of great potential use to Canadian Forces and the first responder community.

Snodgrass, M., Dickinson Laing, T., Mah, D.C., Lam, V., and Aw, C. 2006. Development and Characterization of a Suspension Bead Array Immunoassay for Ovalbumin. DRDC Suffield CR 2006-248. Defence R&D Canada – Suffield.

Table of contents

List of figures

List of tables

Introduction

Exposure to biological threat (BT) agents has recently emerged as a potential threat to the civilian populace as well as the military. The dispersal of anthrax through the US mail in 2001 showed that such events can quickly become a reality [1]. A larger concern may be for First Responders and public health personnel, who may face a greater risk during a release or outbreak of a BT agent due to their exposure to patients, samples, or environments containing one or more pathogens. Members of the Canadian Forces (CF) may encounter BT agents intentionally, by opposing combatants in biological warfare tactics, or by accident, through naturally occurring sources. For example, exposure to *Burkholderia pseudomallei* (the causative agent of melioidosis), could occur during deployment in overseas locales, such as during the tsunami relief effort in southeast Asia in 2005. These CF members infected by a BT agent could be in areas which lack physicians with the knowledge to diagnose the cause of the illness; or the illness could be caused by a rare pathogen. Initial misdiagnosis could increase the time required for the infected individual to receive the proper treatment, a situation that could have deadly consequences. Therefore, there is a need for the development of a rapid detection system for detection of multiple BT agents that is both sufficiently sensitive and specific to identify a broad range of potential pathogen classes such as bacteria, viruses and toxins.

The enzyme-linked immunosorbent assay (ELISA) is the most widely used method of detection of these pathogens because antibody-based technologies are highly selective, specific, sensitive, and adaptable in identifying BT agents [1, 2]. The ELISA is a robust immunoassay that does not require much sample processing prior to analysis, compared to other laboratory analytical tests such as polymerase chain reaction (PCR) assays [3]. Although the ELISA is the current gold standard for BT agent detection, it has some limitations [4]. One major drawback of the ELISA is that it requires a separate assay for each antigen of interest [2]. Thus, several ELISAs must be performed to test for a variety of BT analytes; this may pose a problem if the sample size is limited. The ELISA methodology requires hours for coating or blocking steps, as well as 30-minute to hour-long incubations after each step of the procedure. Hence, testing by ELISA may be laborious and time-consuming. Another disadvantage of the ELISA is the limited dynamic range of the assay, for example 1 ng/mL to 1000 ng/mL, such that repeated testing with further dilutions of the test sample may be necessary [2]. Testing for BT agents by ELISA also requires a large quantity of specific antibodies for each BT analyte, which can be difficult to produce and expensive to purchase from commercial sources.

A newly-developed alternative to the traditional ELISA is the Luminex liquid suspension array-based immunoassay system. This system employs 5.5 μm polystyrene micro-beads which are embedded with precise ratios of two (red and infrared) fluorescent dyes [3]. Currently, an assortment of 100 bead classes is available, each with a unique spectral signature, to allow for a flow cytometry-based dual laser detector system [3, 5]. The Suspension Array System (SAS) from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, ON) utilizes this Luminex bead technology to permit the multiplexing of up to 100 different assays within a single sample [5]. These beads can be utilized in a sandwich immunoassay format, by covalently coupling each set of beads to a different capture antibody (CAb) (Figure 1).

Beads are then mixed and incubated with the sample in a 96-well filtration plate. To detect each of the captured antigens in the sample, detector antibodies (DAb) that specifically bind to the antigens are added and followed by incubation; indicator antibodies (IAb) labeled with a fluorescent phycoerythrin (PE) reporter tag are then added. For analysis, the contents of each well are drawn into the array reader; precision fluidics aligns the beads in single file through a flow cell where two lasers excite the fluorophores in the beads [5]. The red 635 nm classification laser excites the dyes in each micro-sphere, identifying its unique fluorescent signature [2, 5]. The green 523 nm reporter laser excites the PE reporter molecule associated with the bead, which allows quantitation of the antigen by its fluorescent intensity (FI) [2, 5]. To eliminate bead aggregates from the analysis, the microsphere size is determined by a 90-degree light scatter of the classification laser, so that only individual beads are read by the reader [5], preventing potential false readings caused by more than one type of bead clumping together. High-speed digital signal processors and software record the fluorescent signals simultaneously for each bead, translating the signals into data for each bead-based assay in real time.

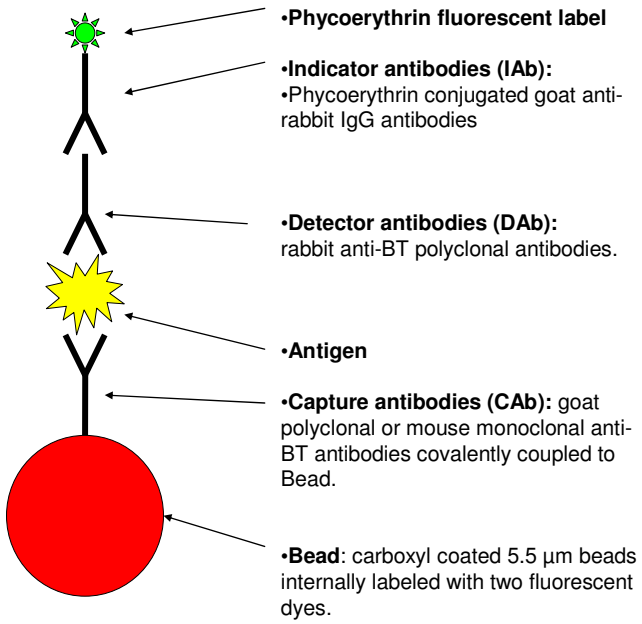


Figure 1. Schematic of a Suspension Array Immunoassay.

The SAS has several advantages over ELISA and other pathogen identification techniques. The most significant advantage is that the SAS can test up to 100 different antigens simultaneously. This conserves the amounts of test sample and reagents required for analysis, as well as saving time required to perform multiple assays for each antigen [4, 6]. The beads used in SAS assays are suspended in solution during incubation, shortening the diffusion path of antigen to antibody binding sites on the beads [1]. Because binding in free solution is more favored kinetically than binding to a solid phase, incubation times can be reduced compared

to ELISA. Thus, SAS assays are rapid and respectable sensitivity can be achieved with incubation times as little as 30 minutes [1]. The antibody concentrations and assay volumes used in SAS are smaller than those used in ELISA, resulting in a lower overall cost per unit assay for SAS assays compared to ELISAs.

Chicken egg albumin (ovalbumin) is the most abundant protein found in chicken egg whites. Ovalbumin is a 45 kDa glycoprotein often used in immunoassays as a simulant for protein toxins such as ricin, botulinum toxin or staphylococcal entertoxins, as a safe alternative for the development of immunoassay protocols. SAS assays have been reported for certain BT agents, including aerosolized *Bacillus anthracis*, *Yersinia pestis*, and botulinum toxoid [7, 8], demonstrating "proof of principle" for this detection approach. However, these reports did not provide a detailed methodology for developing unique assays. By developing and characterizing an ovalbumin SAS assay, our goal was to create a template for the development of SAS assays for other BT agents. In addition, we wished to compare the sensitivity of SAS to ELISA for the detection of ovalbumin.

Materials and Methods

Antigens, Antibodies and Beads

Monoclonal anti-ovalbumin antibody OVA-14 produced in mouse, and anti-chicken egg albumin antibody produced in rabbit, were obtained from Sigma (St. Louis, MO). Rabbit anti-ovalbumin polyclonal antibodies were purchased from Chemicon International Inc. (Temecula, CA). Purified rabbit anti-ovalbumin (FL12) antibodies, PE-conjugated goat F(ab')2 anti-rabbit IgG (H+L), and PE-conjugated goat anti-mouse IgG (H+L) used as SAS assay IAb, were obtained from Cedarlane Laboratories Ltd (Hornby, ON). Anti-mouse IgG (H+L) horseradish peroxidase (HRP) conjugated rabbit antibodies and anti-rabbit IgG (H+L) HRP-conjugated goat antibodies were obtained from Bethyl Laboratories Inc. (Montgomery, TX) and used for ELISA IAb. Albumin from chicken egg white, grade V, minimum 98% was obtained from Sigma. COOH Bead 010 was purchased form Bio-Rad Laboratories Inc. (Hercules, CA).

Buffers and Reagents

ELISA coating buffer (0.05 M carbonate-bicarbonate pH 9.6), ELISA wash buffer (phosphate buffered saline, pH 7.4 (PBS), 0.1% bovine serum albumin (BSA), 0.1% Tween-20), ELISA blocking buffer (PBS, 2% BSA) were used in ELISA. Diluent buffer (PBS, 2% BSA, 0.1% Tween-20) was used for dilutions of antibodies, antigens and beads. PBS-BSA (PBS, 1% BSA) was used for all washing and dilutions of antibodies and antigens in the SAS assays. Carbonate-bicarbonate (0.05 M, pH 9.6) capsules, PBS and Tween 20 were obtained from Sigma. BSA, fraction V, was purchased from EMD Chemicals (Gibbstown, NJ). ABTS Peroxidase Substrate System (peroxidase substrate solution A and peroxidase substrate B), obtained from KPL (Gaithersburg, MD), was used in ELISA for the HRP reaction. All buffers used in antibody purification (ImmunoPure Immobilized Protein G Plus gel slurry, Binding Buffer and Elution Buffer) were provided in a NAb™ Spin Purification Kit obtained from Pierce (Rockford, IL). All buffers used in antibody coupling to beads (bead wash buffer, bead activation buffer, PBS buffer, bead blocking buffer, bead storage buffer and staining buffer) were provided in an Amine Coupling Kit from Bio-Rad Laboratories Inc. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (S-NHS) used in the antibody coupling to beads, was obtained from Pierce.

ELISA

CAb was diluted in ELISA coating buffer to 5-15 µg/mL and 100 µL was added to each well of a Nunc Maxisorb 96-well flat bottom plate. The plate was incubated overnight at 4°C. Wells were washed five times with ELISA wash buffer and 300 µL of ELISA blocking buffer was added to block open, protein-binding sites on the plate. The plate was incubated for 1 hour at 37°C and then washed five times with ELISA wash buffer. The plate was then blocked again for 1 hour at 37°C with ELISA blocking buffer. Following five more washes with ELISA wash buffer, the antigen was diluted with ELISA diluent buffer; 100 µL of the

antigen solution was added to the antigen positive wells, while 100 µL of ELISA diluent buffer was added to the remaining wells. The plate was incubated for 1 hour at 37°C and then washed five times with ELISA wash buffer. An aliquot (100 µL) of DAb diluted in ELISA diluent buffer were added to the wells. The plate was incubated for 1 hour at 37°C and washed five times in ELISA wash buffer, followed by the addition of 100 µL of anti-DAb IAb conjugated with HRP diluted in ELISA diluent buffer. The plate was incubated for 1 hour at 37°C and washed five times with ELISA wash buffer. Next, 200 µL of ABTS HRP substrate solution was added to all wells and incubated for 30 minutes at room temperature in the dark. Absorbance of the samples was read at 405 nm in a spectrophotometer. Optimal DAb and IAb concentrations were determined by titration.

ELISA CAb Optimization

CAb diluted in ELISA coating buffer was titrated at concentrations ranging from 3-30 µg/mL; 100 µL of each concentration was added to wells of a Nunc Maxisorb 96-well flat bottom plate. The plates were incubated overnight at 4°C. Wells were washed five times with ELISA wash buffer and 300 µL of ELISA blocking buffer was added. The plate was incubated for 1 hour at 37°C and then washed five times with ELISA wash buffer, followed by a second blocking step for 1 hour at 37°C. Plates were washed five times with ELISA wash buffer. Anti-CAb IAb conjugated with HRP was diluted with ELISA diluent buffer and 100 µL was added to each well. The plate was incubated for 1 hour at 37°C and washed five times with ELISA wash buffer. Next, 200 µL of ABTS HRP substrate solution was added to all wells and incubated for 30 minutes at room temperature in the dark. Absorbance of the samples was read at 405 nm in a spectrophotometer.

Antibody Purification

Antibody purification was performed using the NAb™ Spin Purification Kit (Pierce) following a protocol supplied by the manufacturer. The ImmunoPure Immobilized Protein G Plus gel slurry was mixed and 200 µL was dispensed into a Handee Spin Cup Column (Pierce). The column was placed in a microcentrifuge collection tube. The gel slurry was mixed with 300 µL of Binding Buffer and the uncapped cup/tube assembly was centrifuged at 5000 x g for one minute. The filtrate was discarded and 400 µL of Binding Buffer was added. The uncapped cup/tube assembly was centrifuged at 5000 x g for one minute and the filtrate was discarded. The antibody containing sample was added and the capped cup/tube assembly was agitated for one hour at 4°C. After the incubation, the cup/tube assembly was uncapped and centrifuged at 5000 x g for one minute. The column then was washed three times with 400 µL of Binding Buffer at 5000 x g for one minute. Contents of the spin cup were transferred to a new collection tube and 400 µL of Elution Buffer was added. The cup assembly was agitated for five minutes and then centrifuged at 5000 x g for one minute. Contents of the spin cup were then transferred to a new collection tube, while the filtrate was saved as the first elution fraction. Three more fractions were collected; protein concentrations of each fraction were determined by measuring the relative absorbance of each fraction at 280 nm in a spectrophotometer.

Bead Coupling

Protein G purified monoclonal anti-ovalbumin antibodies were covalently coupled to COOH Bead 010 using the Amine Coupling Kit protocol provided by the manufacturer. The beads were vortexed and sonicated for 30 seconds and a 100 µL aliquot was added to the amine coupling reaction tubes. The tubes were centrifuged at 14000 x g for six minutes at 4°C. Supernatants were removed with a plugged Pasteur pipette. The beads were resuspended in 100 µL of bead wash buffer and centrifuged at 14000 x g for six minutes at 4°C. The supernatants were removed and the beads were resuspended in 80 µL of bead activation buffer. Ten µL of 50 mg/mL EDC was added to each tube followed by 10 µL of 50 mg/mL S-NHS. The tubes were wrapped in foil and agitated at room temperature for 20 minutes. After incubation, 150 µL of PBS was added and the tubes were centrifuged at 14000 x g for six minutes at 4°C. The supernatants were removed and the beads were resuspended in 100 µL of PBS. Different amounts of CAb were added to each reaction tube and the volume was adjusted to 500 µL with PBS, so that the final concentrations of CAb ranged between 0.25-11 µg/mL. The tubes were wrapped in foil and agitated overnight at 4°C. The next day, the tubes were centrifuged at 14000 x g for 6 minutes at 4°C and the supernatants were removed. The beads were washed in 500 µL of PBS and centrifuged at 14000 x g for six minutes at 4°C. The supernatants were removed and the beads were resuspended in 250 µL of bead blocking buffer. The tubes were wrapped in foil and agitated for 30 minutes at room temperature. After blocking, the tubes were centrifuged at 14000 x g for six minutes at 4°C and the supernatants were removed. The beads were washed in 500 µL of PBS and centrifuged at 14000 x g for six minutes at 4°C. The supernatants were removed and the beads were resuspended in 150 µL of Storage Buffer. The tubes were then stored in the dark at 4°C.

Coupled Bead Validation

Aliquots (50 µL) of 1 µg/mL PE-labeled anti-CAb antibody diluted in staining buffer, were added to tubes labeled “Test”; 50 µL of staining buffer was added to tubes labeled “Negative”. The coupled beads were vortexed for 15 seconds; 10000 beads were added to both the “Test” and “Negative” labeled tubes. The tubes were covered in foil and agitated for 30 minutes at room temperature. The beads were centrifuged at 14000 x g for six minutes at 4°C and the supernatants were removed. The beads were resuspended in 125 µL of storage buffer and transferred to a 96-well flat bottom plate. The plate was placed in the 100 SAS reader, where 50 µL of each sample well were analyzed for a median FI from 100 coupled beads.

SAS Assay

Assays were conducted in 96-well filtration plates, pore size 1.2 µm, from Millipore (Bedford, MA). An aliquot (50 µL) containing 5000 coupled beads was mixed with 50 µL of sample; the filtration plate was then shaken at 500 rpm for 30 minutes at room temperature in the dark. The mixture was vacuum aspirated, washed four times with 100 µL of PBS-BSA to remove unbound antigen, and the beads were resuspended in 50 µL of PBS-BSA. DAb (Rabbit anti-

ovalbumin polyclonal antibody) aliquots (50 µL) were added to the wells; the filtration plate was then shaken at 500 rpm for 30 minutes at room temperature in the dark. The mixture was vacuum aspirated, washed four times with 100 µL of PBS-BSA to remove excess DAb, and the beads resuspended in 50 µL of PBS-BSA. Fifty µL of IAb (PE-labeled goat anti-rabbit IgG antibody) was added to the wells and the filtration plate was shaken at 500 rpm for 30 minutes at room temperature in the dark. The mixture was vacuum aspirated, washed four times with 100 µL of PBS-BSA, and resuspended in 100 µL of PBS-BSA. The filtration plate was then read in the Bio-Plex 100 SAS reader according to the supplied Bio-Plex suspension array system hardware instruction manual (4006205 Rev C). Fifty µL of sample from each well was analyzed to determine the median FI from 100 coupled beads. Optimal DAb and IAb concentrations were determined by titration.

Results and Discussion

Development and Optimization of an Ovalbumin ELISA

The first step in developing an ELISA sandwich immunoassay was to determine which antibody combination had the highest signal-to-noise ratio. To determine which anti-ovalbumin antibodies would make the best capture and detector antibodies, an indirect capture ELISA was performed using all three anti-ovalbumin antibodies as capture and detector antibodies in different combinations (Figure 2). Using the rabbit anti-ovalbumin polyclonal antibody as the CAb1 and the Sigma mouse anti-ovalbumin monoclonal antibody as the DAb (DAb2), produced a low absorbance reading. This was especially noticeable for the Chemicon rabbit anti-ovalbumin polyclonal antibodies (Figure 2). Using the Sigma mouse anti-ovalbumin monoclonal antibody as the capture antibody (CAb2) and the rabbit anti-ovalbumin polyclonal antibody as the DAb (DAb1 or DAb3), produced very high absorbance readings. Using the Sigma rabbit anti-ovalbumin polyclonal antibody as the DAb1 produced a high background signal; however, this high background was not present when using the Chemicon rabbit anti-ovalbumin polyclonal antibodies as the DAb3. The high background observed when using the Sigma rabbit anti-ovalbumin polyclonal antibodies as the DAb was the result of using unpurified antibody; the background was attributed to non-specific and/or cross-reactivity with other, extraneous proteins in the unpurified stock.

The signal-to-noise ratios were the most relevant data examined when determining the optimum CAb and DAb combination for ELISA (Table 1). The signal-to-noise ratio, when Sigma rabbit anti-ovalbumin polyclonal antibody was used as Dab, was less than 2 because of its high background; this antibody was not selected as the DAb. In contrast, the signal-to-noise ratio was between 11 and 15 when Sigma mouse monoclonal antibody was used as the CAb (CAb2) and the Chemicon rabbit polyclonal antibody was used as the DAb (DAb3). This combination thus appeared to be the best for ELISA.

However, it could not be assumed that the optimum combination of CAb and DAb antibodies for ELISA was also the optimum combination for the SAS assay. It was necessary to determine whether switching the DAb and CAb in the SAS format would result in higher observed signal-to-noise ratios. Before coupling CAb to the beads, the Sigma mouse anti-ovalbumin monoclonal antibody required further purification to prevent low signal and higher background from contaminating proteins that might be present in the ascites fluid.

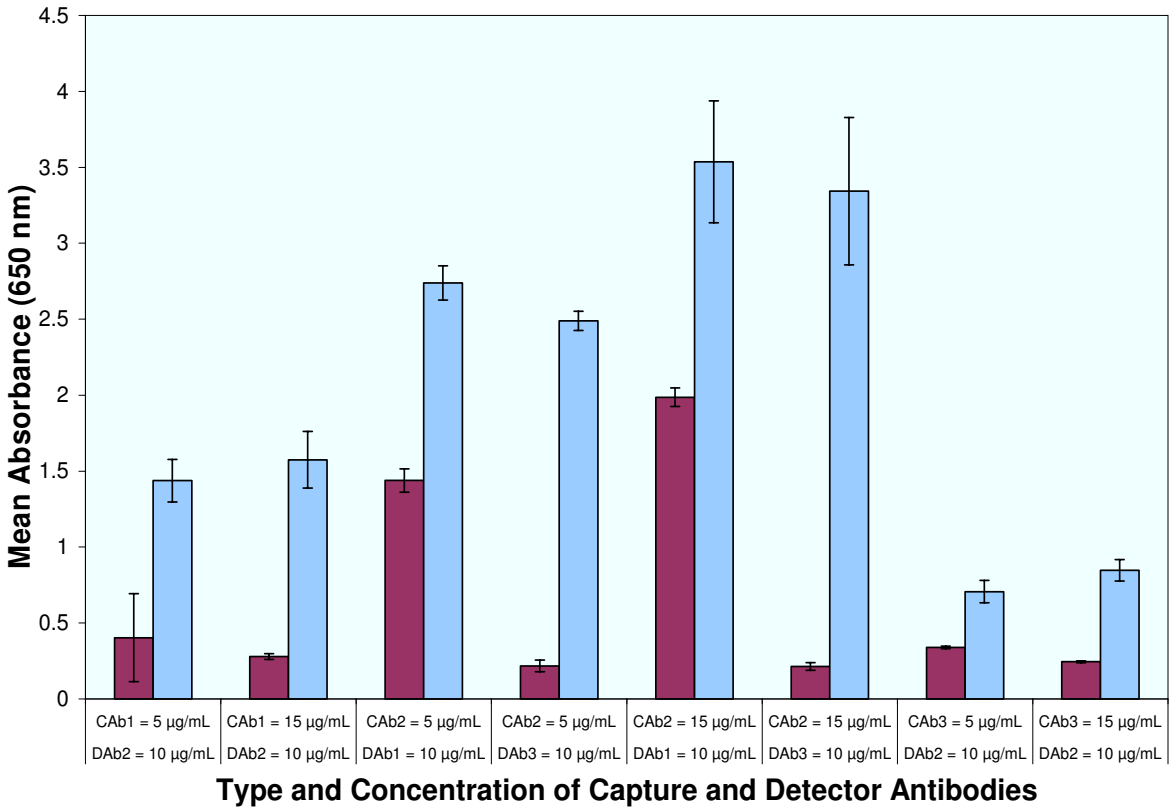


Figure 2. Optimization of Indirect Ovalbumin ELISA.

No Antigen Negative Control Ovalbumin = 10 µg/mL

CAb1 and DAb1 = Sigma rabbit anti-ovalbumin polyclonal. CAb2 and DAb2 = Sigma mouse anti-ovalbumin monoclonal. CAb3 and DAb3 = Chemicon rabbit anti-ovalbumin polyclonal. TMB used as HRP substrate.

Table 1. Comparison of Signal-to-Noise Ratios for Different Combinations of Capture and Detector antibodies in ELISA

Type and Conc. of CAb	DAb Type and Conc.	Average Sample Absorbance	Background Absorbance	Signal-to-Noise Ratio
CAb1 = 5 µg/mL	DAb2 = 10 µg/mL	1.43737	0.4033	3.564
CAb1 = 15 µg/mL	DAb2 = 10 µg/mL	1.57457	0.2795	5.6342
CAb2 = 5 µg/mL	DAb1 = 10 µg/mL	2.73813	1.4385	1.9035
CAb2 = 5 µg/mL	DAb3 = 10 µg/mL	2.4892	0.2176	11.438
CAb2 =15 µg/mL	DAb1 = 10 µg/mL	3.53587	1.9864	1.7801
CAb2 =15 µg/mL	DAb3 = 10 µg/mL	3.34333	0.2141	15.613
CAb3 = 5 µg/mL	DAb2 = 10 µg/mL	0.70627	0.3399	2.0779
CAb3 = 15 µg/mL	DAb2 = 10 µg/mL	0.84697	0.2448	3.4603

The Sigma monoclonal antibody was purified by the NAb™ Spin Purification kit to determine if purification of this antibody would decrease the background and/or increase the signal.

When the unpurified and purified antibody, were compared in ELISA it was observed that the purified Sigma mouse capture antibody (CAb) produced an average increase in absorbance of over 60%, compared to the unpurified CAb (Figure 3). The protein G purified DAb did not significantly increase the signal, but rather produced a significantly higher background absorbance. Thus, the NAb™ Protein G Spin Purification Kit appeared not to be effective in purifying the Sigma anti-ovalbumin antibodies to reduce background noise.

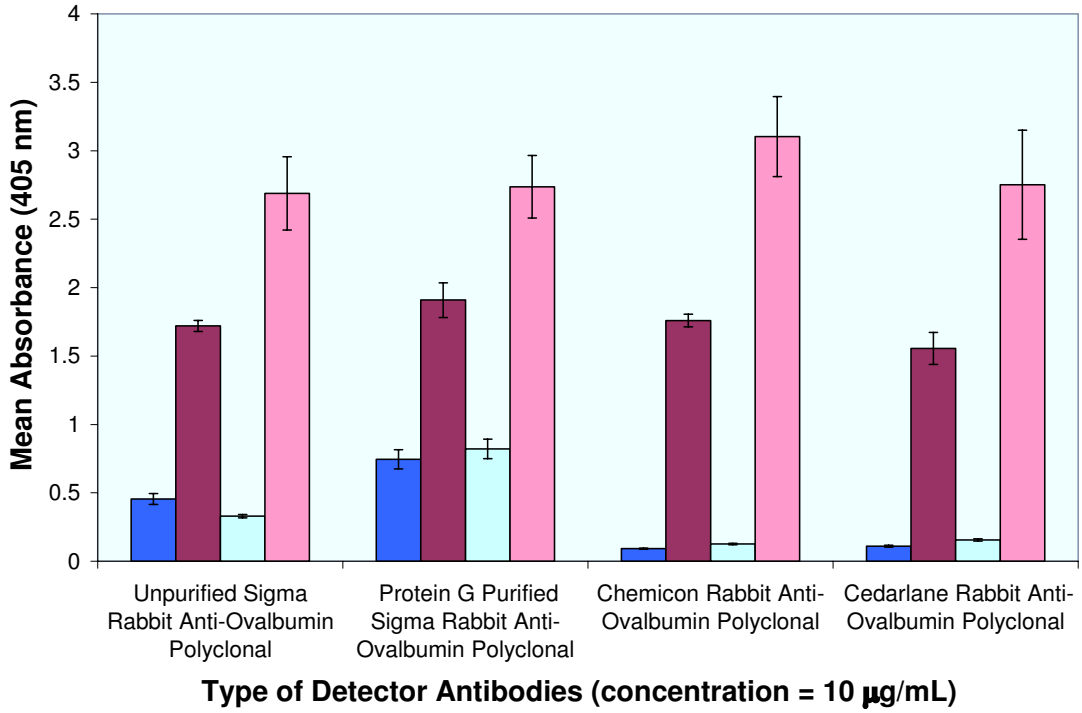


Figure 3. Optimization of Detector Antibodies in Indirect Ovalbumin ELISA.

No Antigen Control: unpurified Sigma mouse anti-ovalbumin monoclonal CAb = 5 µg/mL and IAb = 60 ng/mL. Ovalbumin = 10 µg/mL, unpurified Sigma mouse anti-ovalbumin monoclonal CAb = 5 µg/mL and IAb = 60 ng/mL. No Antigen Control: Protein G purified Sigma mouse anti-ovalbumin monoclonal CAb = 5 µg/mL and IAb = 60 ng/mL. Ovalbumin = 10 µg/mL, Protein G purified Sigma mouse anti-ovalbumin monoclonal CAb = 5 µg/mL and IAb = 60 ng/mL.

A fourth DAb, affinity purified rabbit anti-ovalbumin polyclonal antibody from Cedarlane, was also included in this experiment. Although the affinity purified Cedarlane DAb produced typical absorbance readings, its observed signal-to-noise ratio was lower than that of the Chemicon DAb (Table 2). The optimal combination thus appeared to be the protein G purified Sigma mouse monoclonal antibody as the CAb and the Chemicon rabbit polyclonal antibody as the DAb.

Table 2. Signal-to-Noise Ratio Comparison between Purified and Unpurified Antibodies.

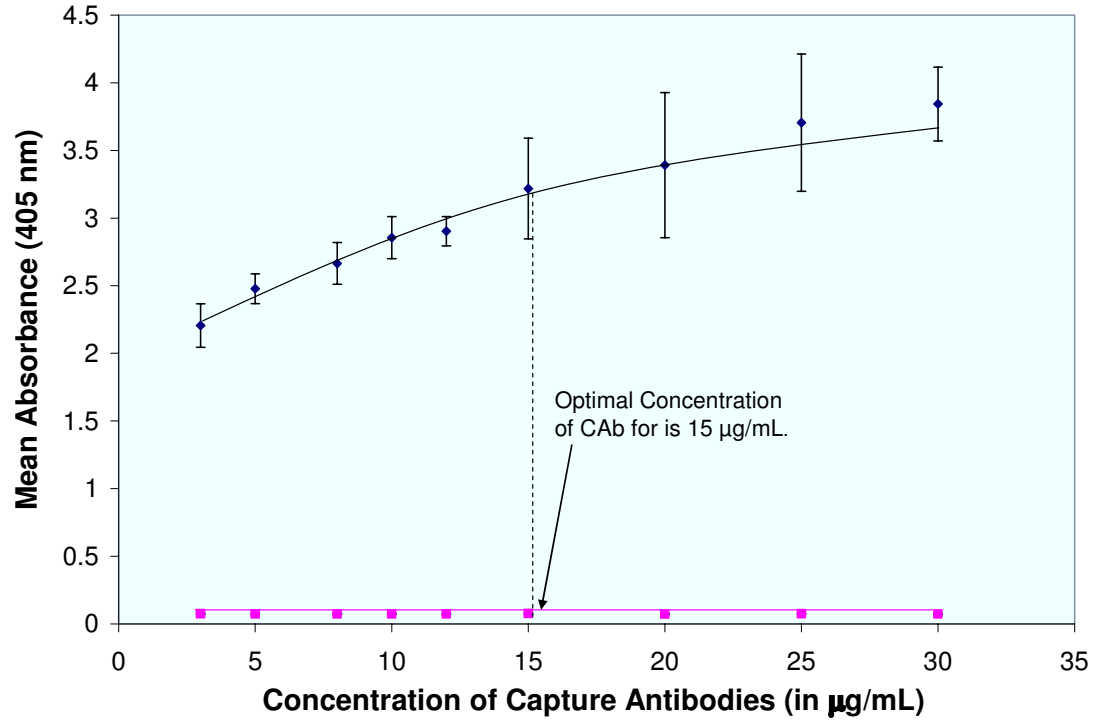
Type of CAb	Type of DAb	Average Sample Absorbance	Background Absorbance	Signal-to-Noise Ratio
CAb1	DAb1	1.7201	0.455	3.7808
CAb1	DAb2	1.9088	0.745	2.5621
CAb1	DAb3	1.7597	0.092	19.127
CAb1	DAb4	1.5554	0.1106	14.059
CAb2	DAb1	2.6889	0.3299	8.1506
CAb2	DAb2	2.7372	0.8208	3.3347
CAb2	DAb3	3.1038	0.126	24.64
CAb2	DAb4	2.7516	0.1564	17.594

CAb1 = Unpurified Sigma mouse anti-ovalbumin monoclonal antibodies
CAb2 = Protein G purified Sigma mouse anti-ovalbumin monoclonal antibodies
DAb1 = Unpurified Sigma rabbit anti-ovalbumin polyclonal antibodies
DAb2 = Protein G purified Sigma rabbit anti-ovalbumin polyclonal antibodies
DAb3 = Chemicon rabbit anti-ovalbumin polyclonal antibodies
DAb4 = Cedarlane rabbit anti-ovalbumin polyclonal antibodies
All CAbs concentration = 5 µg/mL, Antigen concentration = 10 µg/mL,
All DAbs concentration = 10 µg/mL, IAb Concentration = 60 ng/mL

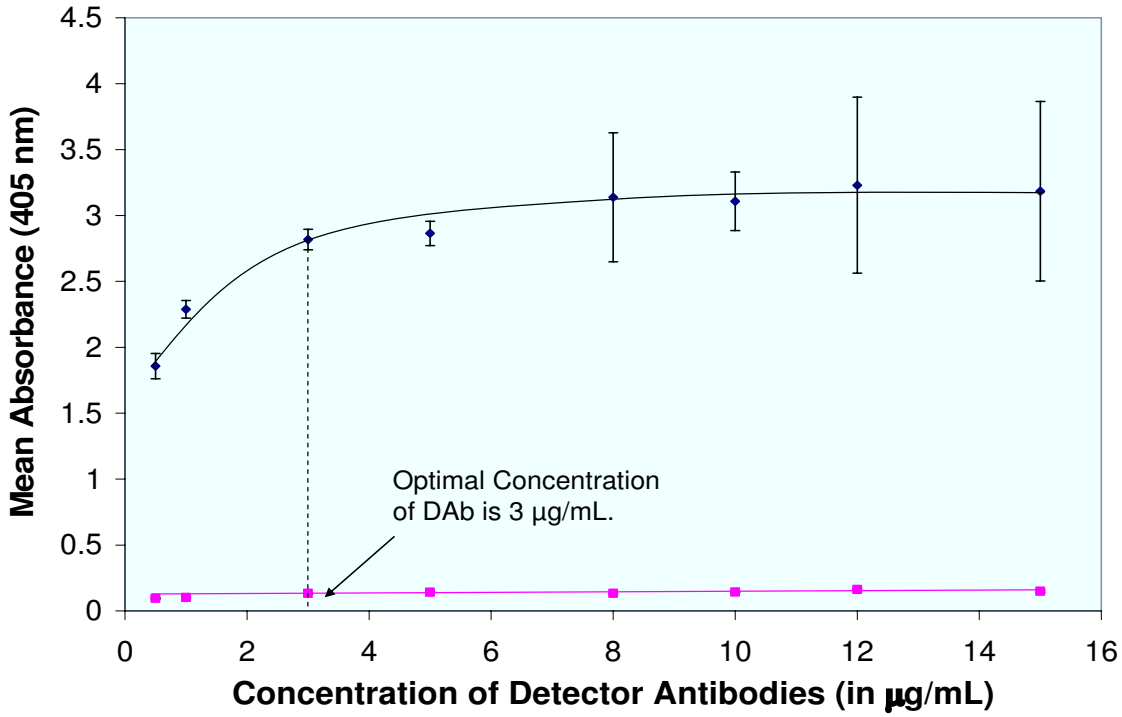
Optimization of CAb, DAb and IAb concentrations for indirect ELISAs was performed using varying concentrations of each test antibody. The optimal concentration of CAb and DAb, defined as the lowest concentration of antibody yielding the maximum absorbance, was found to be 15 µg/mL (Figure 4a) and 3 µg/mL (Figure 4b), respectively. Using these optimized concentrations, the optimal concentration of the IAb was 80 ng/mL (Figure 4c).

To determine the LOD of the optimized indirect capture ovalbumin ELISA, an antigen titration was performed using 2-fold serial dilutions of ovalbumin from 39.1 ng/mL to 4.8 pg/mL. The LOD was defined as the lowest antigen concentration giving an absorbance reading higher than two standard deviations above the mean background absorbance. Using this approach, the LOD was 9.5 pg/mL (Figure 4d). This LOD was 30 fold more sensitive than the LOD of 300 pg/mL for ovalbumin obtained by McBride et. al. [1]. This difference in LODs may have been due to the use of two different antibodies for capture and detection in our assay, compared to the use of the same antibody for capture and detection by McBride and colleagues [1].

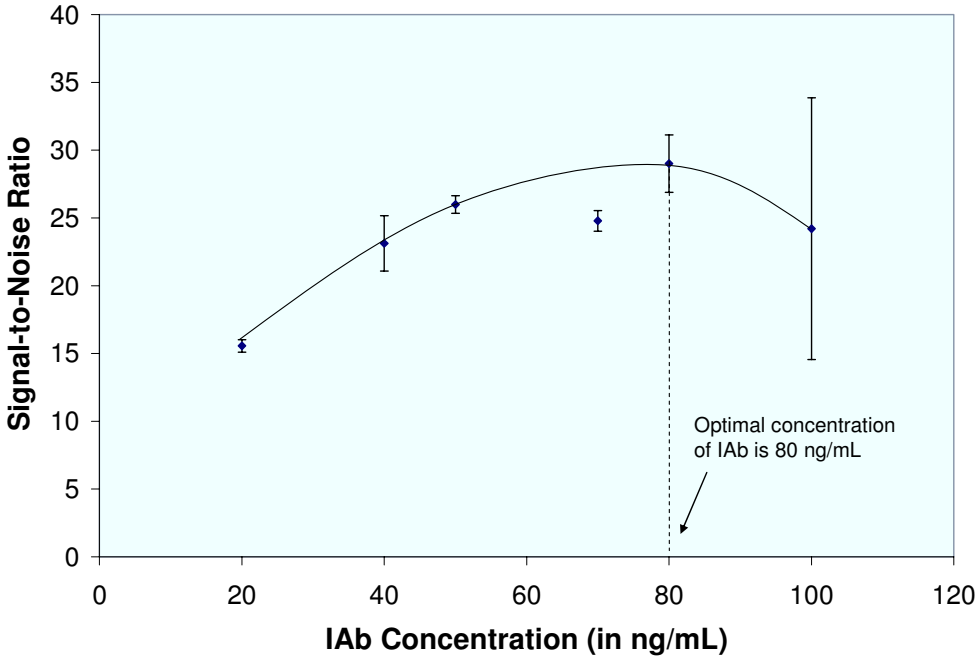
a)


Mean Absorbance (405 nm)
Concentration of Capture Antibodies (in µg/mL)
Optimal Concentration
of CAb for is 15 µg/mL.

b)


Mean Absorbance (405 nm)
Concentration of Detector Antibodies (in µg/mL)
Optimal Concentration
of DAb is 3 µg/mL.

c)



d)

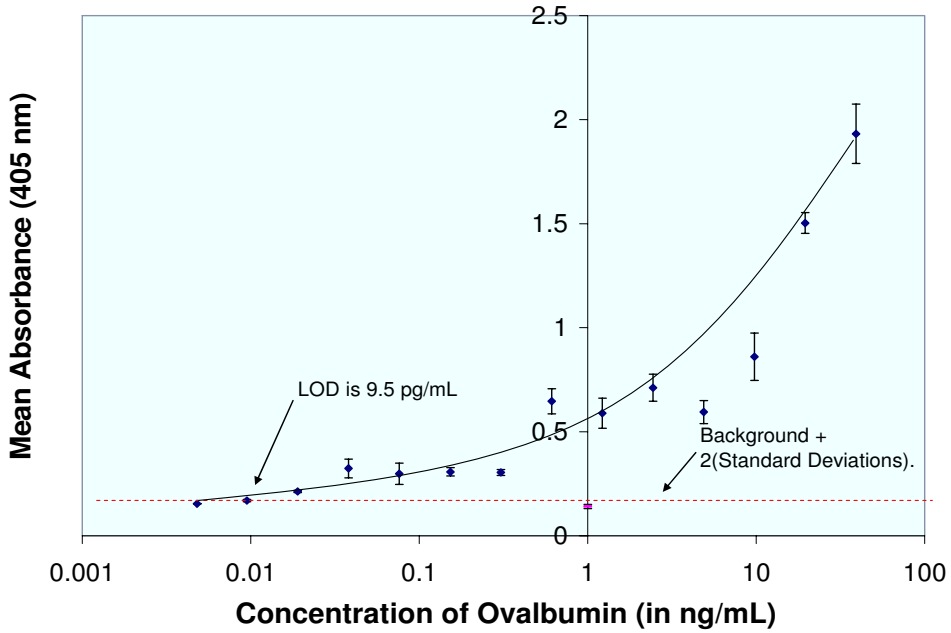


Figure 4. Indirect Capture Ovalbumin ELISA optimization

a) Optimization of CAb in an indirect ovalbumin ELISA. ♦ IAb = 0.2 µg/mL ■ No IAb b) Optimization of DAb in an indirect ovalbumin ELISA. ♦ Ovalbumin = 5 µg/mL, CAb = 15 µg/mL and IAb = 60 ng/mL. ■ No Antigen Control, CAb = 15 µg/mL and IAb = 60 ng/mL. c) Optimization of IAb in an indirect ovalbumin ELISA. Ovalbumin = 10 µg/mL, CAb = 15 µg/mL and DAb = 3 µg/mL. d) LOD determination of an indirect ovalbumin ELISA CAb = 15 µg/mL, DAb = 3 µg/ml and IAb = 80 ng/mL.

Ovalbumin Suspension Array Immunoassay Development

Optimization of Antibody Coupling to Beads

Protein G purified Sigma mouse anti-ovalbumin monoclonal CAb was coupled to bead region 10 using protein concentrations from 0.25 µg/mL to 11 µg/mL, to determine the optimal concentration needed for coupling. After the coupling process, bead validations were performed to determine the optimal concentration of CAb to couple to the beads. It was observed that the optimal CAb coupling concentration was 5 µg/mL, which was determined by the lowest concentration of CAb yielding the highest fluorescent intensity (FI); however, all other concentrations of coupled CAb, with the exception of 0.25 µg/mL, also yielded FI values over the bead validation cut off of 10000 FI (Figure 5). It was concluded that these beads had enough CAb coupled to them to perform a SAS assay. However, the performance of these latter beads remains to be tested, in order to determine whether lower protein concentrations may be used for coupling.

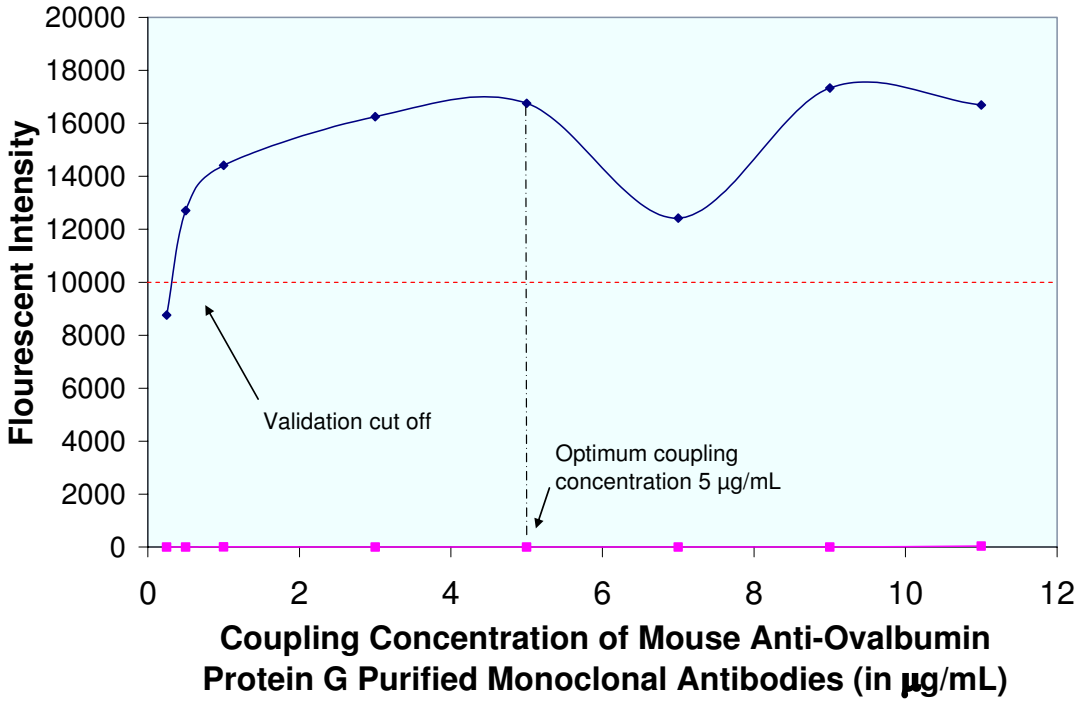


Figure 5. Coupling Validation of Mouse Anti-Ovalbumin Protein G-Purified Monoclonal Antibodies to Suspension Array Beads.

♦ 1 µg/ml of PE conjugated goat anti-mouse IgG DAb. ■ No PE conjugated goat anti-mouse IgG DAb.

Of note was the observation of a noticeable decrease in the FI value at the 7 µg/mL coupling concentration. This was a repeated observation over five bead validations with two different couplings. At this antibody concentration, it was possible that dimerization of antibodies occurred, which might have prevented optimal binding to the beads. Alternatively, this observation may simply have reflected an artifact of the coupling procedure.

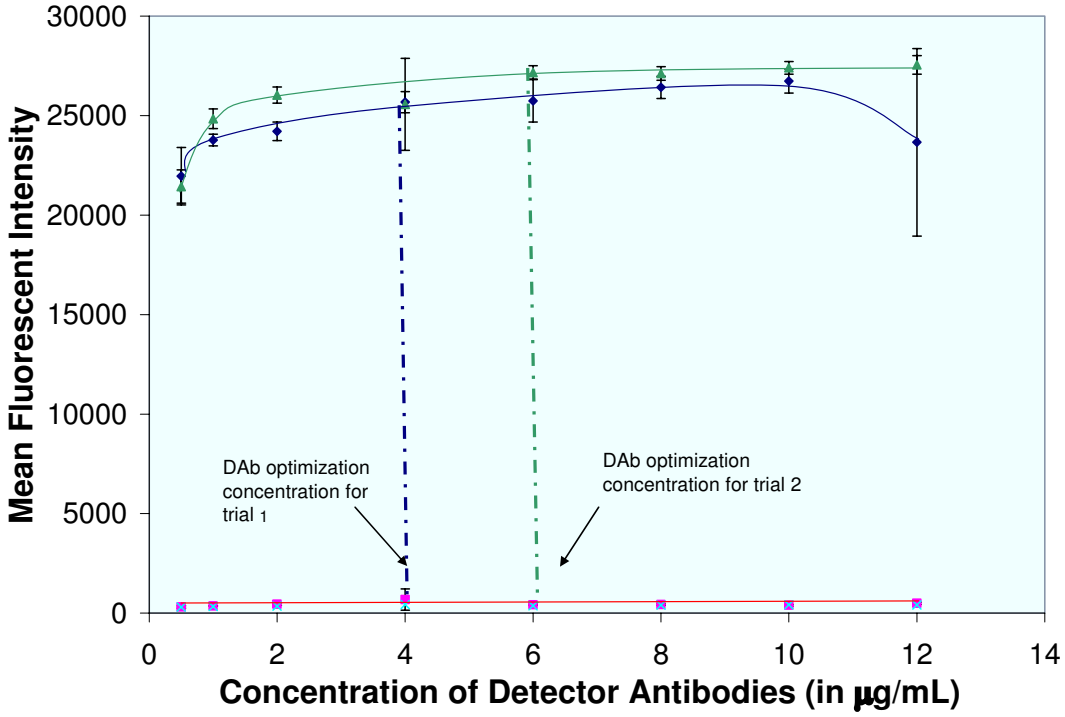
SAS Assay Optimization

A SAS assay was performed with the optimized coupled beads, using concentrations of Chemicon rabbit anti-ovalbumin polyclonal DAb ranging from 0.5 µg/mL to 12 µg/mL to determine the optimal concentration of DAb. The IAb, goat anti-rabbit IgG conjugated PE (Cedarlane), was in excess at a constant concentration of 10 µg/mL. The concentration of ovalbumin used in the antigen positive wells was 10 µg/mL. Under these conditions, the lowest concentration of DAb that produced the highest FI was 4 µg/mL, as determined by the optimization curve in Figure 6a. When this experiment was repeated, the lowest concentration of DAb that produced the highest FI was 6 µg/mL. It was determined that the concentration of DAb used in the future experiments should be the mean of optimized concentrations. Thus, the optimal concentration of anti-ovalbumin detector antibodies was set at 5 µg/mL.

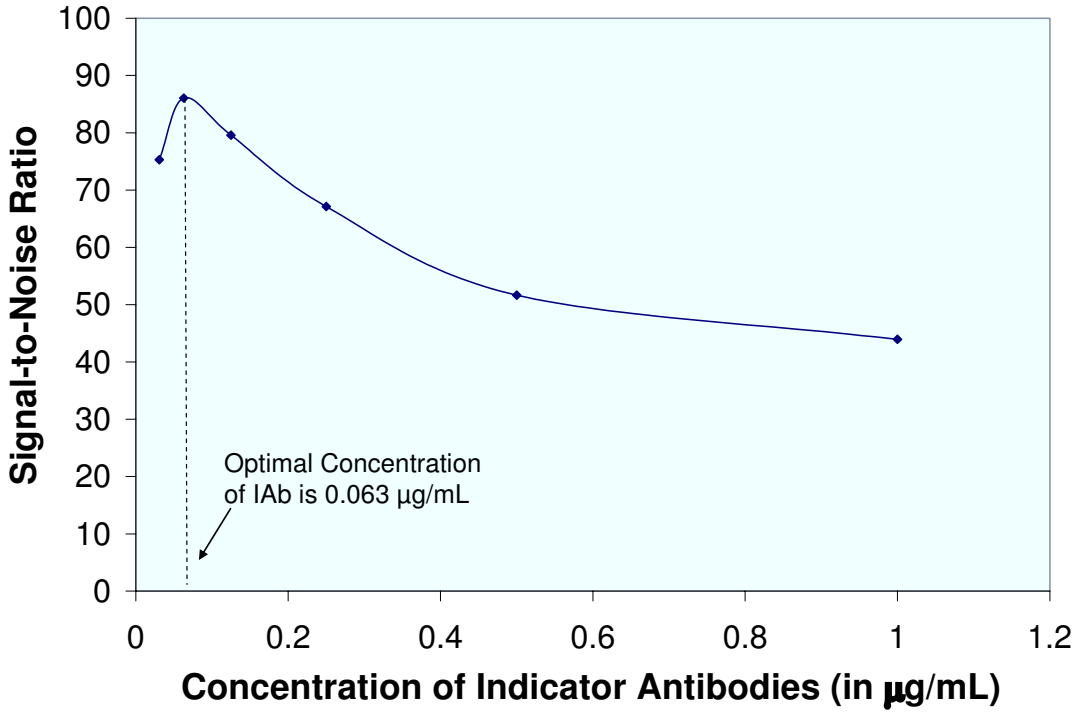
The optimal concentration of IAb was determined by a SAS anti-ovalbumin assay with concentrations of goat anti-rabbit IgG PE-labelled antibodies ranging from 0.031 to 1 µg/mL, using the optimized DAb at 5 µg/mL and antigen excess at 10 µg/mL. A curve relating IAb concentration to the signal-to-noise ratio was constructed (Figure 6b) to determine the optimal IAb. The optimal concentration of IAb was defined as the concentration which yielded the highest signal-to-noise ratio. As the concentration of IAb decreased the signal-to-noise ratio increased. The maximal signal-to-noise ratio occurred at 0.063 µg/mL IAb, but this concentration had a low signal strength of 7298.4 FI compared to higher concentrations of IAb that had signal strengths over 20000 FI (Figure 6c). Thus, 0.25 µg/mL was chosen as the optimal concentration of IAb because it was the lowest concentration of IAb that had a signal strength over 20000 FI, while still maintaining a high signal-to-noise ratio.

Using these optimized parameters, an antigen titration was performed on ovalbumin to determine the LOD of the indirect ovalbumin immunoassay, using 2-fold serial dilutions from 312 ng/mL to 0.038 ng/mL. The LOD, defined as the lowest ovalbumin concentration that had a FI higher than two standard deviations above the background FI, was 4.88 ng/mL (Figure 6d).

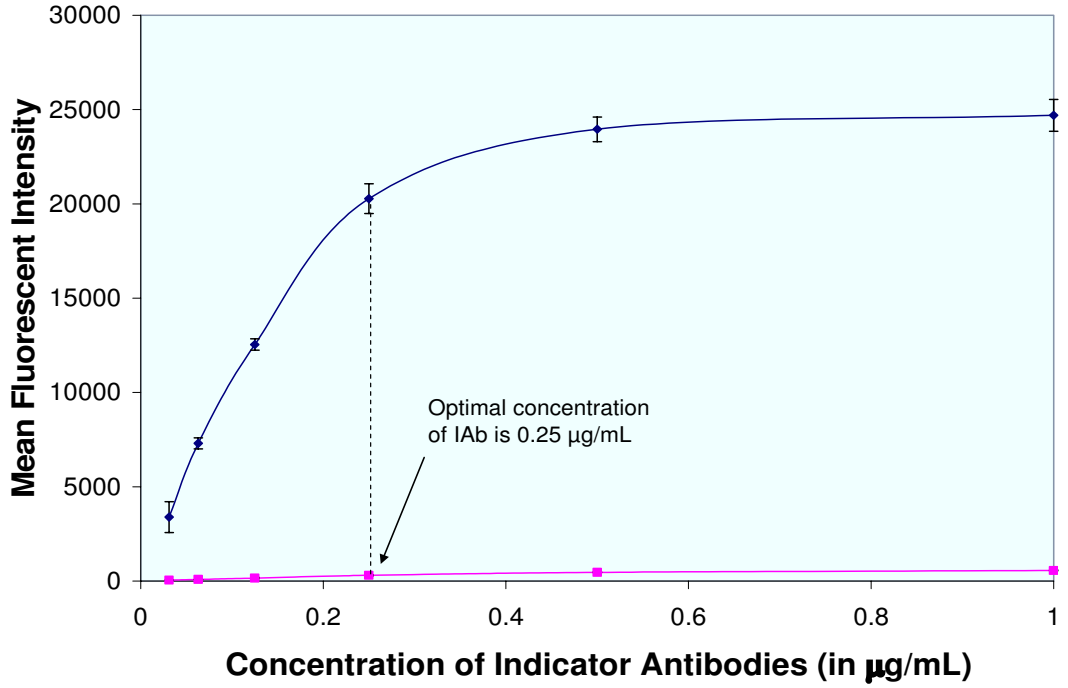
a)


30000
25000
20000
15000
10000
5000
0
Mean Fluorescent Intensity
DAb optimization concentration for trial 1
DAb optimization concentration for trial 2
0
2
4
6
8
10
12
14
Concentration of Detector Antibodies (in µg/mL)

b)


100
90
80
70
60
50
40
30
20
10
0
Signal-to-Noise Ratio
Optimal Concentration of IAb is 0.063 µg/mL
0
0.2
0.4
0.6
0.8
1
1.2
Concentration of Indicator Antibodies (in µg/mL)

c)


Mean Fluorescent Intensity
30000
25000
20000
15000
10000
5000
0
0
0.2
0.4
0.6
0.8
1
Optimal concentration
of IAb is 0.25 µg/mL
Concentration of Indicator Antibodies (in µg/mL)

d)

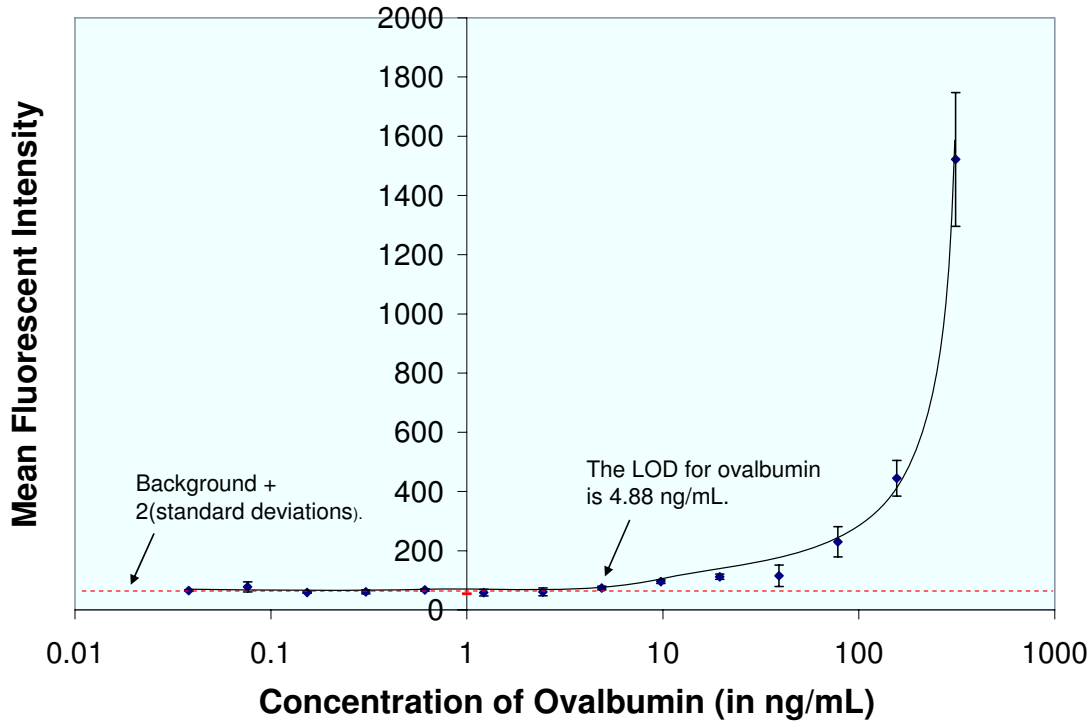

Mean Fluorescent Intensity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0.01
0.1
1
10
100
1000
Background +
2(standard deviations).
The LOD for ovalbumin
is 4.88 ng/mL.
Concentration of Ovalbumin (in ng/mL)

Figure 6. SAS assay optimization

a) Optimization of DAb in a SAS immunoassay. ♦▲ Ovalbumin and IAb = 10 µg/mL. x ■ No ovalbumin, IAb = 10 µg/mL b) Optimization of IAb in a SAS immunoassay. Ovalbumin = 10 µg/mL and DAb = 5 µg/mL. c) Optimization of IAb in a SAS immunoassay. ♦ Ovalbumin = 10 µg/ml and DAb = 5 µg/ml. ■ No antigen, DAb = 5 µg/ml. d) LOD determination of a SAS immunoassay DAb = 5 µg/mL and IAb = 0.25 µg/mL.

When comparing the LODs of ovalbumin for ELISA (9.5 pg/mL) and the SAS assay (4.88 ng/mL), the ELISA was more than 500-fold more sensitive than the SAS assay (Table 3). In a previous study by McBride et. al. [1], a ELISA LOD of 300 pg/mL and a SAS assay LOD of 1 ng/mL was reported; this SAS assay was approximately 5-fold more sensitive than our bead-based assay, with a corresponding 30-fold difference in the ELISA LODs. The difference between the observed LODs of both the ELISA and SAS immunoassays may have been because we used an indirect immunoassay format, whereas McBride and colleagues used a direct immunoassay format. Also, it is possible that some antibody inactivation had occurred during the coupling step, due to chemical crosslinking of the antigen binding site; this may have resulted in the decreased sensitivity observed for the SAS format.

Table 3. Comparison of LODs for ELISA and SAS assays.

Antigen	SAS Assay LOD	ELISA LOD
Ovalbumin	4.88 ng/mL	9.5 pg/mL

Important parameters to assess for the SAS assay were variability of the data, and day-to-day reproducibility. With the standard SAS assays that have been performed thus far, the average per cent coefficient of variance (%CV) of the median FI from one sample well to the next was 5.56% over four wells (Table 4). Meanwhile, the %CV of the average FI from one plate to the next was 14.2% for four assay plates run over 22 days. In comparison, the ELISA assay had a well-to-well %CV of 4.9% over three wells and the average plate-to-plate %CV was 14.8% over three assay plates in a 8 day time span (Table 4). Hence well-to-well and plate-to-plate reproducibility appeared to be similar for both ELISA and SAS technologies.

Table 4. Well-to-well and Plate-to-Plate Variability

Type of Assay	# of Assay Plates	# of Wells per Assay	Average Signal	Minimum Signal	Maximum Signal	Average Background	Well-to-Well %CV	Plate-to-Plate %CV
SAS	4	4	21328 FI	18586.5	23570	194	5.56%	14.2%
ELISA	3	3	2.5844 AU	1.7865	3.0045	0.1062	4.90%	14.8%

Standard condition for SAS: CAb = 5 µg/mL, ovalbumin = 10 µg/mL, DAb = 5 µg/mL and IAb = 0.25 µg/mL. Standard condition for ELISA: CAb = 15 µg/mL, ovalbumin = 10 µg/mL, DAb = 3 µg/mL and IAb = 80 ng/mL.

Conclusion

In conclusion, a SAS assay for ovalbumin was developed, which produced rapid, sensitive and reproducible results. The procedures we developed to characterize the ovalbumin assays can now be used to develop other SAS assay for BT agents such as toxins, bacteria and viruses. Once these SAS assays are developed, they can be used to create a multiplex assay, which will then be used to test for several BT agents simultaneously from a single sample.

References

1. McBride M.T., Gammon, S., Pitesky, M. O'Brien, T.W., Smith T., Aldrich, J., Langlois, R.G., Colston, B. and Venkateswaran, K.S. (2003). Multiplexed liquid arrays for simultaneous detection of simulants of biological warfare agents. *Anal. Chem*., 75, 1924-1930.

2. Pang, S., Smith, J., Onley, D., Reeve, J., Walker, M. and Foy, C., (2005). A comparability study of the emerging protein array platforms with established ELISA procedures. *J. Immunol. Meth.,* 302, 1-12.

3. Rao, R.S., Visuri, S.R., McBride, M.T., Albala, J.S., Matthews, D.L. and Coleman, M.A. (2004). Comparison of multiplexed techniques for detection of bacterial and viral proteins. *J. Proteome Res*., 3, 636-742.

4. Mandy, F.F., Nakamura, T., Bergeron, M. and Sekiguchi, K. (2001). Overview and application of suspension array technology. *Clinics Lab Med.,* 21 (4), 713-729.

5. Fulton, R.J., McDade, R..L., Smith, P.L., Kienker, L.J. and Kettman, J.R. Jr. (1997). Advanced multiplexed analysis with the FlowMetrix™ system. *Clin. Chem*., 43 (9), 1749-1756.

6. Nolan, J.P. and Mandy, F.F. (2001). Suspension array technology: new tools for gene and protein analysis. *Cell. Mol. Biol*., 47 (7), 1241-1256.

7. McBride, M.T., Masquelier, D., Hindson, B.J., Makarewicz, A.J., Brown, S., Burris, K., Metz, T., Langlois, R.G., Tsang, K.W., Bryan, R., Anderson, D.A., Venkateswaran, K.S., Milanovich, F.P. and Colston B.W. Jr. (2003). Autonomous detection of aerosolized *Bacillus anthracis* and *Yersinia pestis.* *Anal. Chem*., 75, 5293-5299.

8. Hindson, B.J., McBride, M.T., Makarewicz, A.J., Henderer, B.D., Setlur, J.S., Smith, S.M., Gutierrez, D.M., Metz, T.R., Nasarabadi, S.L., Venkateswaran, K.S., Farrow, S.W., Colston, B.W, Jr. and Dzenitis, J.M. (2004). Autonomous detection of aerosolized biological agents by multiplexed immunoassay with polymerase chain reaction confirmation. *Anal. Chem*., 77, 284-289.

List of symbols/abbreviations/acronyms/initialisms

%CV	Per cent coefficient of variance
BSA	Bovine serum albumin
BT	Bio-threat
CAb	Capture antibody
CF	Canadian Forces
DAb	Detector antibody
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assay
FI	Fluorescent intensity
HRP	Horseradish peroxidase
IAb	Indicator antibody
LOD	Limit of detection
PBS	Phosphate buffered saline
PE	Phycoerythrin
SAS	Suspension array system
S-NHS	N-hydroxysulfosuccinimide

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.)

Canada West Biosciences Inc.
5429 60th Street
Camrose, AB T4V 4G9

2. SECURITY CLASSIFICATION
(overall security classification of the document, including special warning terms if applicable)

Unclassified

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title).

Development and Characterization of a Suspension Bead Array Immunoassay for Ovalbumin

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

Snodgrass, M., Dickinson Laing, T., Mah, D.C., Lam, V., and Aw, C.

5. DATE OF PUBLICATION (month and year of publication of document)

December 2006

6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) 27

6b. NO. OF REFS (total cited in document) 8

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Contract Report

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

DRDC Suffield, PO Box 4000 Stn Main, Medicine Hat, AB, T1A 8K6

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)

9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

W7702-04-R044/001/EDM

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)

DRDC Suffield CR 2006-248

10b. OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

(x) Unlimited distribution
( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
( ) Distribution limited to government departments and agencies; further distribution only as approved
( ) Distribution limited to defence departments; further distribution only as approved
( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

Unlimited

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

The Suspension Array System (SAS) has enabled the development of assays that are rapid, sensitive and specific for any biological threat agent. In this study a SAS immunoassay was developed and characterized for the toxin simulant ovalbumin. Optimization of this assay and its performance are presented. Results obtained were compared with data obtained by ELISA. The limit of detection (LOD) for the SAS ovalbumin assay was 4.88 ng/mL, compared to a LOD of 9.5 pg/mL for the ovalbumin ELISA. When compared to the LODs for ovalbumin reported in a previous study, the difference between the assay sensitivities was considerably smaller, with an ELISA LOD of 300 pg/mL and a SAS assay LOD of 1 ng/mL. The 500-fold sensitivity difference was likely a reflection of the longer incubation times for the ELISA versus the SAS assays, where the incubation time for the ELISA was 1 hour compared to an incubation time of 30 minutes for the SAS. The trade off for this decrease in sensitivity is a rapid assay that can be performed in half the time as a traditional ELISA. The SAS ovalbumin assay had a well-to-well variance of 5.56%, while its plate-to-plate reproducibility was 14.2%. In comparison, the ELISA ovalbumin assay had a well-to-well variance of 4.90%, whereas its plate-to-plate reproducibility was 14.8%. In summary, an immunological assay that was rapid, sensitive and reproducible was created for a biological threat simulant by suspension array technology. The procedures used to develop the SAS assay for ovalbumin can be used as a template for development of other assays for toxins, bacteria, and viruses. SAS assays for individual biothreat agents can, in turn, be used as a starting point for the development of multiplexed assays for multiple agents.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Immunoassay
ELISA
Suspension Array
Ovalbumin