

CREST Program - Final Technical Report

Rapid Sensitive Water Quality Assessment with an Immunosensor

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Elizabeth R. Abboud, Interdisciplinary Graduate Program in Molecular and Cellular Biology

Diane A. Blake, Ph.D., Mentor and Professor of Biochemistry

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Tulane University Health Sciences Center, Department of Biochemistry

1430 Tulane Avenue, SL-43

New Orleans, LA 70112

Note: Submission of this final technical report was delayed due to the damage suffered by our laboratory in the aftermath of Hurricane Katrina

Pollution of the Gulf of Mexico due to human and animal waste creates significant problems for the inhabitants of coastal communities. The negative impact of fecal contamination demands increased efforts to control this problem. The primary goal of this high risk, high reward project was to develop a rapid, sensitive, species-specific immunosensor to monitor the severity of fecal contamination in environmental water sources while the problem is under management.

Immunoglobulin A (IgA) is present in mucosal secretions and hence is secreted in the feces. Previous studies from other laboratories have shown that levels of IgA in wastewater correlate well with coliform contamination. The development of an assay that could quantify levels of IgA from different species would allow regulatory agencies to monitor not only the extent of fecal contamination in waters, but also the source of the contamination.

The principal objectives of this project were to develop an assay system that was sensitive enough to detect environmentally-relevant levels of IgA present in wastewater and surface effluents, and to adapt the assay system to the prototype handheld immunosensor under development in our laboratory.

Year 01 activities:

- Commercially available antibodies to human IgA were surveyed for their ability to perform in a sandwich ELISA assay for IgA.
- Large quantities of a rabbit polyclonal antibody to human IgA were generated in-house.
- IgA-specific IgG was purified from this polyclonal antiserum.

Year 02 Activities:

- Sandwich ELISA reagents were adjusted to increase sensitivity.
- Sensitivity of the capture antibodies in the sandwich ELISA for IgA was compared using commercially available and in-house generated antibodies.
- Rabbit IgG fractions were pooled and protein concentrations were measured for each sample.
- The pooled rabbit IgG fractions were compared for their ability to bind to IgA.
- The pooled rabbit IgG fractions and goat polyclonal to IgA were compared as capture reagents in the sandwich ELISA.
- The ELISA sandwich assay for IgA was adapted to the immunosensor format.
- Varying the capture reagent, reagent concentration and flow rate failed to provide the sensitivity sufficient to monitor IgA levels in environmental water sources.

NEW FUNDING LEVERAGED AS A RESULT OF THIS PROJECT:

GRO Graduate Fellowship: Awarded to Elizabeth R. Abboud in July 2005 by the U.S. Environmental Protection Agency (\$117,000, one of 16 awarded nationally)

PUBLICATIONS THAT ACKNOWLEDGE CREST FUNDING:

D.A. Blake, R.C. Blake II, E.R. Abboud, X. Li, H. Yu, A. M. Kriegel, M. Khosraviani, and I.A. Darwish (2006) "Antibodies to heavy metals: Isolation, characterization and incorporation into microplate-based assays and immunosensors". In *Immunoassay and Other Bioanalytical Techniques* (Ed. J.M. Van Emon), Taylor and Francis, Boca Raton, FL, in press.

E.R. Abboud (2007) Selection and Characterization of High-Affinity Synergistic Antibodies from a Phage Display scFv Immune Library, Ph.D. Thesis, Tulane University, *in preparation*.

RATIONALE FOR EXPERIMENTS PERFORMED

The “sandwich” immunoassay format we used for this assay required that the IgA have at least two distinct sites where antibodies could bind. The first antibody in the sandwich was immobilized on a solid surface and served to “capture” the IgA; the second antibody then bound to IgA at the second site, and signal was generated by addition of an enzyme-labeled secondary antibody that bound to the second antibody in the sandwich (see Figure 1). In the sandwich format, the sensitivity of the assay depends upon both the strength of the antibody binding to IgA (antibody affinity) and the amount of first and second antibody used in the assay (total number of antibody binding sites, Elkins, 1998). As the affinity of the antibodies in the assay increases, so does the chance that the limited molecules of IgA in the environmental sample will bind to the antibody. Increasing the concentration of antibody binding sites would also be expected to increase the final sensitivity in the assay. In the experiments performed during this CREST-funded project, we attempted to increase both antibody affinity and the number of antibody binding sites available in the assay.

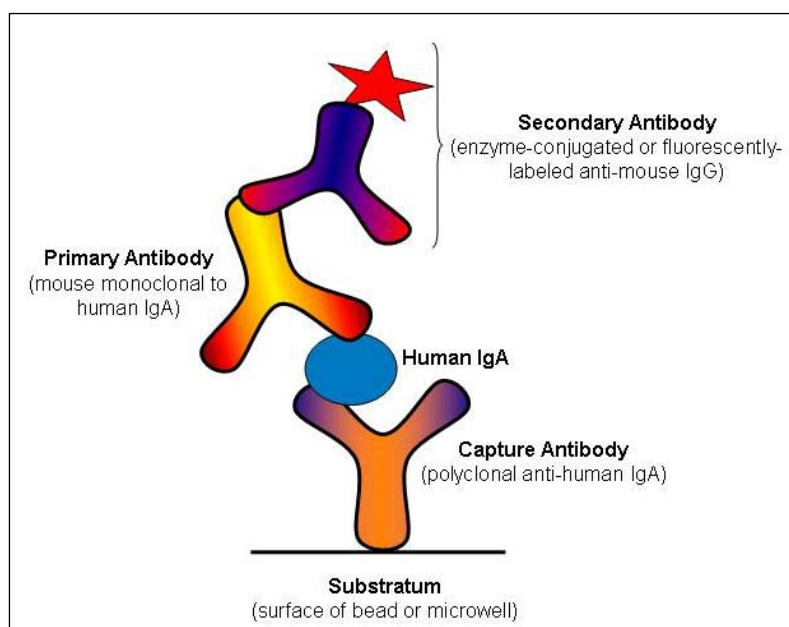


Figure 1. Schematic diagram of the sandwich immunoassay. Microwell plate (ELISA format) or beads (immunosensor format) were coated with saturating concentration of a polyclonal capture antibody. Following blocking of non-specific binding sites with BSA, standard or sample containing IgA was allowed to bind to the immobilized capture antibody. Bound IgA was detected by sequential incubation of a monoclonal primary antibody and an enzyme-conjugated or fluorescently-labeled secondary antibody. Signal was generated by the addition of either TMB substrate or activation of the fluorescent label.

OVERVIEW OF YEAR 01 ACTIVITIES

Preparation and characterization of antibody reagents and development of a sandwich ELISA for human IgA

We surveyed a wide variety of commercially available antibodies to IgA to determine which bound with highest affinity. These experiments indicated that none of the commercially available antibodies were capable of detecting low concentrations of IgA that were expected in environmental water samples. Thus, research in the first year of this project focused upon improving the quality of antibody reagents, which would in turn enhance the sensitivity of the final antibody-based assay. We chose to prepare custom antibodies to IgA by immunizing rabbits with IgA and collecting the polyclonal antisera. We purified large amounts of rabbit serum antibodies to IgA and tested them in the existing immunoassay. We also attempted to increase the number of antibody binding sites in the assay by purifying from the serum only those antibodies that bound specifically to human IgA. However, the polyclonal antibodies prepared via these methods provided only minor improvements in sensitivity (30% or less) as compared to the commercially available antibodies.

OVERVIEW OF YEAR 02 ACTIVITIES

Further refinement of the sandwich ELISA, adaptation of the sandwich assay to the immunosensor format, and comparison of sensitivity of the two formats

In the second year of the project, we made some changes to the reagents used in the sandwich assay in a final effort to increase the sensitivity before moving to the sensor format. We first compared the binding activity of three different enzyme-labeled anti-species antibodies and incorporated the one with the lowest non-specific binding into the assay. We then compared the capture abilities of the commercial and in-house preparations of polyclonal antibodies to IgA in the assay using the new secondary antibody. Purified rabbit IgG fractions were pooled for each animal and protein concentrations were measured for each sample. The different IgG fractions were also compared for their ability to bind to IgA. Finally, the capture ability of each pooled rabbit IgG preparation was compared to the polyclonal goat antibody to IgA.

In our experiments to adapt this assay to the sensor format, we varied the nature of the bead used to immobilize the capture antibody (polystyrene versus polymethylacrylate versus Azlactone), varied the flow conditions in the sensor assay and increased the concentrations of the all antibodies used in the assay. Despite our efforts to maximize the sensitivity of the sensor-based assay, the sensor-based assay was 30-fold less sensitive than our best sandwich ELISA. We were unable to get our range of detection below approximately 500 ppb, and we expect that the environmental concentrations of IgA would be much lower (low parts per billion to parts per trillion).

To some extent, our efforts to maximize sensitivity may have been thwarted by the lower surface area available for immobilization of the capture antibody in the immunosensor format. The total surface area available for immobilization of the capture antibody in the sandwich ELISA format assay was approximately 0.6 cm² (based on manufacturers' specifications). The total surface area available for immobilization of the capture antibody in the immunosensor format was 0.23 cm² (Blake, Pavlov and Blake, 1999). Clearly, some of the decrease in sensitivity was due to the decreased surface area available for immobilization of the capture antibody, and further experiments to increase sensitivity in this format will include design of a new capture cartridge that will have a larger surface area available for immobilization of the capture antibody. We may also try to increase the density of capture antibody on the bead surface by using biotinylated dendrimers. Each immobilized dendrimer can be derivatized with at least 10 capture antibodies.

The 3-fold lower surface area for the immobilization of capture antibodies is not sufficient, however, to explain the 30-fold lower sensitivity we observed in the sensor format, and we postulate that further loss of sensitivity in this format was due to the flow-based system used in the sensor format. In the sandwich ELISA, the IgA remains in contact with the capture antibody for 60 minutes. In the flow-based sensor we used for these analyses, each IgA molecule is only in contact with the immobilized capture antibody for ~1-2 seconds, even at the slowest flow rate programmable in the sensor. In future experiments, we may pre-incubate the beads containing immobilized capture antibody with the sample containing IgA, and then load then into the cassette for final analysis. This would increase the time required to perform the assay, but should serve to increase sensitivity in the final assay. In the final analysis, however, we may discover that the sensor under development in our laboratory is better suited for the analysis of low molecular weight contaminants like heavy metals and marine toxins than for large protein analytes, such as IgA.

DETAILED TECHNICAL REPORT

I. Sandwich ELISA reagents were surveyed in an effort to increase sensitivity.

- **Rationale:** We attempted to decrease the background signal in our sandwich ELISA by using different enzyme-linked anti-species antibodies for detection. We compared the non-specific binding of three commercially available antibodies to mouse IgG (our primary detection antibody in the sandwich assay).
- **Results:** Donkey anti-mouse IgG (H+L) displayed the least non-specific binding to all three of the coating antibodies.

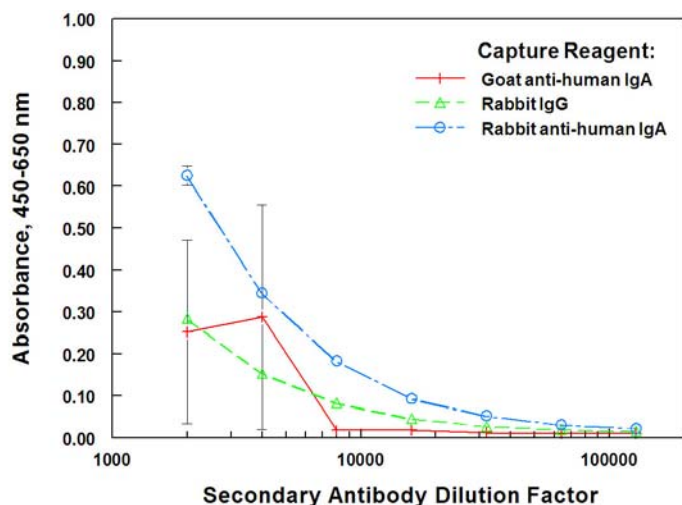


Figure 2. Comparison of non-specific binding activity of three different secondary antibodies in the sandwich ELISA. Plates were coated with 2.5 $\mu\text{g}/\text{mL}$ of one of three polyclonal capture antibodies: goat anti-human IgA (Jackson), rabbit IgG fraction or rabbit anti-human IgA (both prepared in-house). One of three peroxidase-conjugated anti-species secondary antibodies was serially diluted on the immobilized capture reagents (donkey anti-mouse IgG (H+L), goat anti-mouse IgG (H+L) and rabbit anti-mouse IgG (H+L), Jackson). Signal was generated with the addition of TMB colorimetric substrate (KPL) and absorbance readings were taken in a spectrophotometer.

II. Sensitivity of the capture antibodies in the sandwich ELISA for IgA was compared using commercially available and in-house generated antibodies.

- **Rationale:** We wanted to determine which of our available coating antibodies could capture the most IgA in the sandwich ELISA. We compared the commercially available goat polyclonal to IgA to our rabbit IgG fraction.
- **Results:** Comparison of the EC_{50} s for the various capture reagents (goat = 32.63 ng/mL and rabbit = 23.79 ng/mL) revealed that while the activities of the goat and rabbit antibodies were similar, rabbits provided a small (30%) increase in sensitivity.

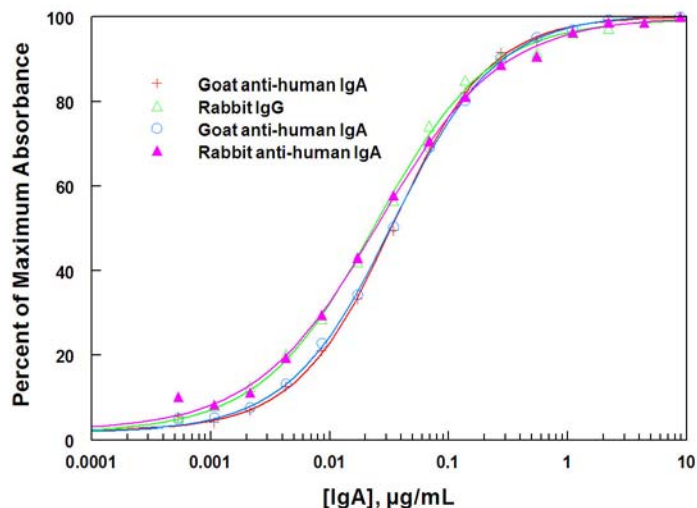


Figure 3. Sandwich ELISA to compare the capture abilities of the three polyclonal antibodies for IgA. Plates were coated with 2.5 $\mu\text{g}/\text{mL}$ of one of three polyclonal capture antibodies: goat anti-human IgA, rabbit IgG fraction or rabbit anti-human IgA. Human IgA was serially diluted on the immobilized capture antibodies. Bound IgA was detected with a mouse monoclonal antibody to human IgA (ab433, abcam) followed by a peroxidase-conjugated anti-species secondary antibody (donkey anti-mouse IgG (H+L), Jackson). Signal was generated with the addition of TMB colorimetric substrate (KPL) and absorbance readings were taken in a spectrophotometer.

III. Rabbit IgG fractions were pooled and protein concentrations were measured for each sample.

- **Rationale:** We wanted to maintain consistency between immunoassays, so we decided to pool each animal's purified IgG. We measured the protein concentration of each sample so that we would be able to use a specific concentration of antibody as (opposed to a dilution factor) in our experiments.
- **Results:** We purified a total of 420 mg of rabbit IgG and the concentrations ranged from 5.68 mg/mg/mL to - 7.60 mg/mg/mL.

Rabbit Number	Volume Pooled, mL	Concentration of Pooled IgG, mg/mL	Total IgG Purified, mg
26	6	6.61	39.66
27	8	5.68	45.44
28	24	7.60	182.4
29	23	6.62	152.26

Table 1. Rabbit IgG fractions were pooled and protein concentration was measured using a BCA assay (Pierce). Standards (bovine gamma globulin) and samples (rabbit IgGs) were diluted to the appropriate concentrations (standard concentrations were 1, 0.5, 0.25, 0.125, 0.625, 0.3125, 0.015625 and 0 mg/mL; samples were 1:10, 1:100 and 1:1,000 dilutions of stock) and working reagent was added to the diluted samples. Samples were incubated at 37°C for 45 minutes and absorbance was read at 562 nm. Bovine gamma globulin was used to generate a standard curve, and the concentrations of the rabbit IgG samples were calculated based on the linear regression for the standard curve.

IV. The pooled rabbit IgG fractions were compared for their ability to bind to human IgA.

- **Rationale:** Before incorporating one of the rabbit IgG fractions into the sandwich assay, we wanted to determine which bound to IgA with the highest affinity.
- **Results:** All of the rabbit IgG preparations performed equally in the sandwich ELISA format.

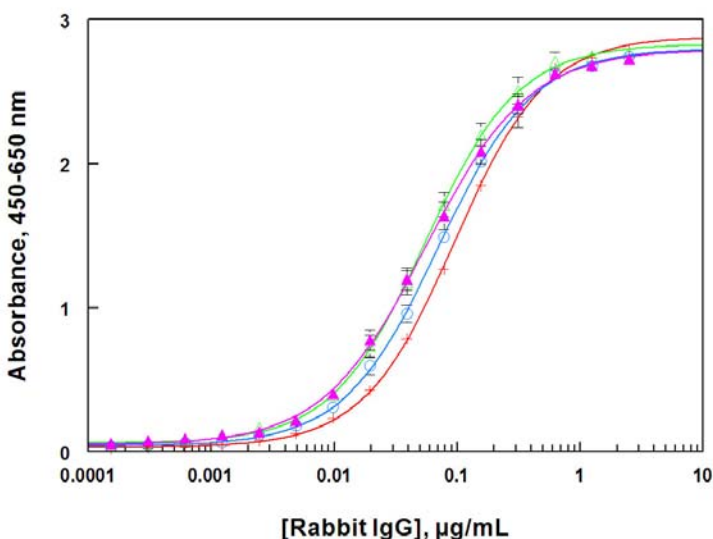


Figure 4. Rabbit IgG fractions were compared for binding activity to IgA. Plates were coated with 2.5 µg/mL of human IgA. Rabbit IgGs were serially diluted on the immobilized IgA. Bound rabbit IgG was detected with a peroxidase-conjugated anti-species secondary antibody (goat anti-rabbit IgG, Jackson). Signal was generated with the addition of TMB colorimetric substrate (KPL) and absorbance readings were taken in a spectrophotometer.

V. The pooled rabbit IgG fractions and goat polyclonal to IgA were compared as capture reagents in the sandwich ELISA.

- **Rationale:** In order to assure that we were using the reagents with the highest sensitivity for IgA in the assay before moving to the handheld format, we compared the capabilities of rabbit IgG fractions and the goat polyclonal to IgA as capture reagents.
- **Results:** The rabbit IgGs did not significantly increase the sensitivity of the sandwich ELISA for IgA.

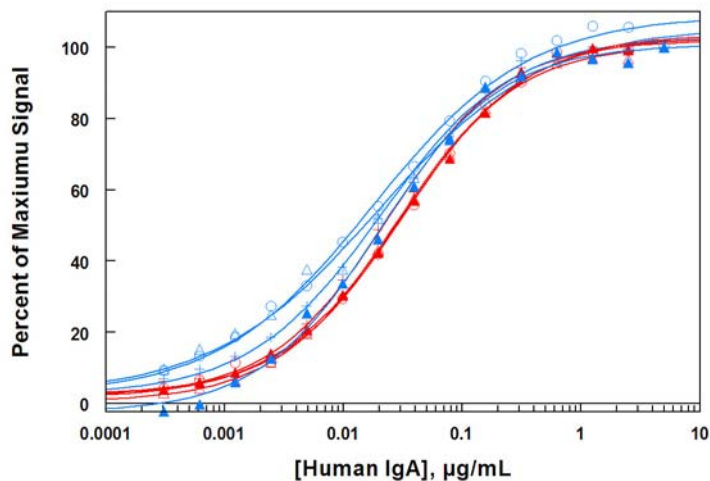


Figure 5. The coating capabilities of the rabbit IgG fractions were compared to the goat polyclonal to IgA in the sandwich ELISA. Plates were coated with 2.5 µg/mL of goat anti-human IgA and each rabbit IgG. Human IgA was serially diluted on the capture antibodies. Bound IgA was detected with a mouse monoclonal antibody to human IgA followed by peroxidase-conjugated anti-species secondary antibody. Signal was generated with the addition of TMB colorimetric substrate (KPL) and absorbance readings were taken in a spectrophotometer. Rabbit IgGs are shown in blue and goat anti-human IgA is shown in red.

VI. The ELISA sandwich assay for IgA was adapted to the immunosensor format.

- **Rationale:** The ultimate goal of this project was to adapt the sandwich assay for IgA to a handheld immunosensor format. At the time these experiments were performed, the handheld sensor was undergoing repairs and could not be used for adaptation of the assay. The KinExA is a larger, but comparable instrument (in format) to the handheld sensor, so we began to transfer the plate-based assay to the KinExA.
- **Results:** Our limit of detection for the assay was low, at 500 ppb IgA. The EC₅₀ for this assay was also higher than the ELISA format, at 970 ng/mL (approximately 30-fold higher).

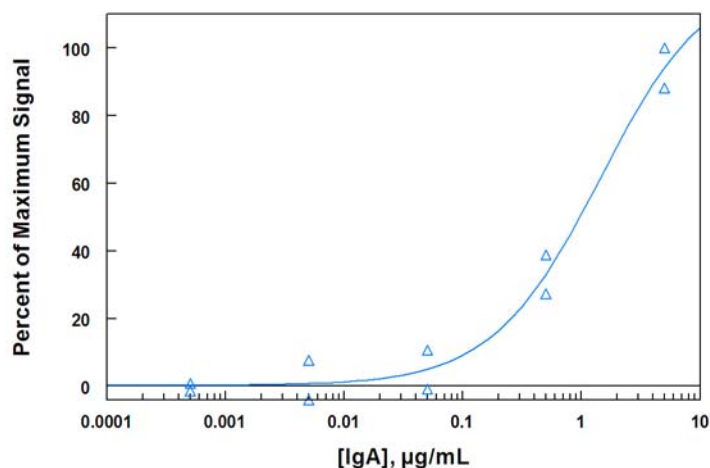


Figure 6. The sandwich assay was tested in the handheld sensor format. Polystyrene beads were coated with 10 µg/mL goat anti-human IgA. Bound IgA was detected with 0.5 µg/mL of mouse monoclonal antibody to human IgA. Signal was generated with a fluorescently-labeled antibody to mouse IgG (1:2,000; Cy-5-labeled donkey anti-mouse IgG, Jackson) flowed over the bead column and measuring fluorescence volts.

- **Rationale:** In order to increase the sensitivity of this assay, we experimented with different the bead types, flow rates, detection antibody concentrations and buffer composition.
- **Results:** We were unable to increase the sensitivity beyond approximately 500 ppb IgA.

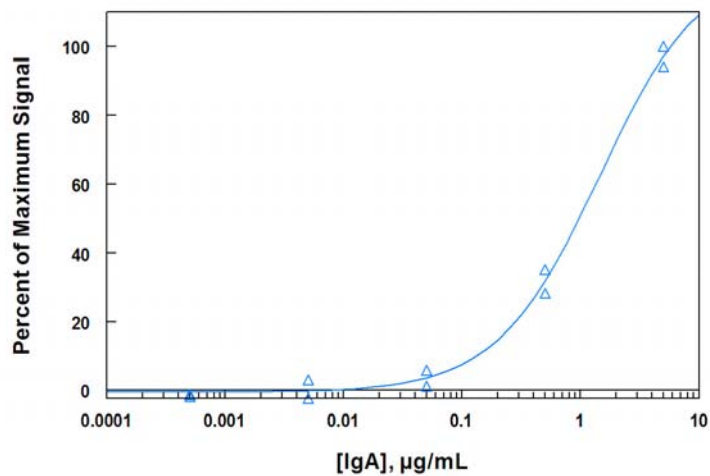


Figure 7. Various assay parameters were adjusted to maximize the sensitivity and signal capabilities. Polystyrene beads were coated with 10 µg/mL goat anti-human IgA. Bound IgA was detected with 0.5 µg/mL of mouse monoclonal antibody to human IgA. Signal was generated with a fluorescently-labeled antibody to mouse IgG (1:8,000; Cy-5-labeled donkey anti-mouse IgG, Jackson) flowed over the bead column and measuring fluorescence volts.