"Construction and Evaluation of a Regenerable Fluoroimmunochemical-Based Fiber Optic Biosensor"

DOE/ER/13613--29

DE91 004886

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Synopsis

A microscale fiber optic biosensor that is capable of *in situ* regeneration is described and characterized. By combining recently developed fiber optic sensing technology with a capillary column reagent delivery system, it is possible to perform a variety of benchtop affinity assay procedures repetitively and remotely. The configuration of the sensing chamber at the terminus of the fiber is an important design feature. The construction and operation of the sensor is described and the results of evaluations of the sensor using an antibody/antigen system are presented. Affinity assay steps such as the delivery of solid phase affinity reagents, secondary reagents, and rinse solutions are demonstrated. Sampling is accomplished by mild aspiration. Coefficients of variation (CVs) for these steps are all less than 10. The capability of selectively measuring fluorescently-labeled anti-rabbit-immunoglobulin G (IgG), in the presence of a similar protein, utilizing its immunospecific interaction with rabbit-IgG beads, is demonstrated and exhibits a CV of 6.2. A near linear calibration plot is presented over a concentration range of 0.011 mg ml^{-1} (approximately the limit of detection) to 0.11mg ml^{-1}.

Keyword: Fiber optic; biosensor; fluoroimmunoassay; laser; IgG

The ability to spectrally monitor chemical concentrations remotely and *in situ* has been enhanced with the advent of small diameter optical fibers that transmit light efficiently over long distances and wide spectral regions. Such measurements offer advantages over traditional approaches that involve sampling, then transporting the sample to the laboratory for subsequent analysis. In particular, chemical concentrations can be measured directly in hostile or not easily accessible environments without the chemical or physical alterations of sample composition commonly associated with traditional approaches. Ideally, the measurements are performed continuously without removing the fiber optic "sensor" from the remotely-located sample. Unfortunately, *in situ* measurements can be complicated and generally do not exhibit the analytical sensitivity and selectivity associated with conventional laboratory techniques.

These problems have been addressed with the development of "fiber optic chemical sensors" (FOCS).[1-3] FOCS signals are a result of the interaction of an analyte with a reagent phase. The chemical/physical specificity afforded by the interaction contributes to the selectivity of the measurement; and high sensitivity achieved if the interaction results in a signal that can be detected by laser-excited fluorimetry. The reagent phase is immobilized at the sensing terminus of the optical fiber by a number of means including direct covalent attachment,[4] entrapment in a gel,[5] and containment in an analyte permeable chamber.[6] Traditionally, FOCS have been used to measure small molecules or ions.[7-11] The application of FOCS to the measurement of large molecules has been accomplished recently by using bioaffinity reagent phases.[3] Among the analyte/reagent phase

combinations that have been employed are lectin/carbohydrate,[12,13] enzyme/substrate,[14-16] and antibody/antigen or hapten.[17-23] Our recent work has focused on the use of immunochemical reagent phases to perform remote measurements by competitive-binding[17] and direct[4,6] assay procedures. The specificity of immunochemical recognition (antibody/antigen association constants, K_as, are typically in the range of 10^8-10^{12}) resulted in excellent sensitivity and selectivity in our previous work.

In order for a sensor to be used in a continuous fashion [1] the interaction of the analyte and reagent phase must be rapid and reversible, thereby permitting competitive-equilibrium-binding operation,[24] or [2] the reagent phase reservoir must not be appreciably depleted during the measurement period, or [3] the sensor must be capable of *in situ* regeneration. Many of the sensors that have been described in the literature do not adequately satisfy any of these criteria and are more correctly termed probes. In general, our previous immunochemical-based FOCSs fall into this category. Two approaches to continuous monitoring with immunochemical-based FOCS have been reported. Anderson and Miller utilized a competitive-equilibrium-binding approach to continuously monitor the drug phenytoin with an antibody-based FOCS.[21] Sensor operation was based on homogeneous fluoroimmunoassay principles and involved competition between labeled and unlabeled drug for antibody. We have demonstrated the feasibility of using a "microscale regenerable biosensor", MRB, to perform rapid, repetitive (e.g., pseudo-continuous) measurements of a benzo(a)pyrene metabolite.[25,26] Operation of this sensor is based

on heterogeneous fluoroimmunoassay procedures. Each approach has its advantages and disadvantages. The competitive-equilibrium-binding approach requires a compromise in K_a value. Rapid response favors small values, while sensitivity and selectivity are enhanced with large values.[3] The MRB is versatile and can exploit the advantages of antibodies with large K_as, but operation can be relatively complicated.

The MRB combines typical FOCS instrumentation with a reagent delivery system that employs capillary columns. In principle, the many bioassays that are used extensively in clinical laboratories can be performed remotely, *in situ*, and in very small samples with the MRB. Assay protocols would vary greatly, but generally employ affinity reagents immobilized an solid supports (immunobeads in this work) and involve the addition of secondary reagents and rinse solutions. The principle aim of the work presented herein is to demonstrate the feasibility and evaluate the repeatability of performing isolated and combined affinity assay operations with the MRB. The general design of the MRB is also described. Calibration plots were obtained for a rabbit-immunoglobulin-G/anti-rabbit immunoglobulin-G (rab-IgG/anti-rab-IgG) immunological system. These plots were used to evaluate the response range and sensitivity of the MRB. By rinsing to expel nonspecific interferents (compounds that do not bind to the immobilized affinity reagent), the specificity of the affinity-analyte interaction is exploited to selectively measure analyte. Another advantage of the MRB is that reproducible sampling of analyte can be accomplished by an aspiration procedure. Previously described sensors sampled analyte by passive diffusion, a

procedure that has limited applicability since many analytes exhibit low diffusivities due to their large size or a lack of sensor permeability. Future prospects for the MRB are also briefly discussed in this report.

EXPERIMENTAL SECTION

Materials

Capillary columns used in the MRB were of various sizes ranging from 200 µm to 520 µm i.d. and were purchased from Polymicro Technologies, Inc., Phoenix, Arizona. Fiber optics used in this work were 400 µm core diameter, plastic clad fused silica fibers (QSF-400), supplied by General Fiber Optics, Cedar Grove, NJ. The epoxy was 5 minute epoxy manufactured by Devcon Corp., Danvers, MA. Syringe needles were standard 16 gauge Luer lock variety. A fiber optic/column bundle template was constructed in house from lexan. Stainless steel frits, 5 µm porosity, were obtained from Newmet Krebsoge, Terryville, CN. A Model 7010 Rheodyne HPLC injection valve and Luer lock syringe valves (Mininert syringe valves, catalog no. 654051) were purchased from Alltech Assoc., Deerfield, IL. The injection valve, fitted with a 50 µl injection loop, was used for bead injection. Plexiglass "T" connectors were fashioned in house. The syringe pump, for sample and reagent introduction, was obtained from Sage Instruments, Cambridge, MA (Model 341A). Phosphate buffered saline (PBS), pH 7.4, fluorescein-isothiocyanate (FITC), anti-rabbit-immunoglobulin-G-fluorescein-isothiocyanate (anti-rab-IgG-FITC), fluorophor to protein ratio (F/P) approximately 3, rabbit-immunoglobin-G-fluorescein-isothiocyanate (rab-IgG-FTIC), F/P approximately 4, rabbit-immunoglobulin-G (rab-IgG) (lypholized),

human-immunoglobulin-G-fluorescein-isothiocyanate (human-IgG-FITC), F/P approximately 4, were obtained from Sigma Chemical Co., St. Louis, MO. Human blood serum was donated by a single subject. Immunobeads were prepared by immobilizing on silica beads (5 μm diameter) using a previously reported procedure.[27] Fluorescently labeled Latex beads used in the bead delivery studies were Fluoresbrite plain microspheres (6.29 μm diameter, λ_{ex} = 488 nm, λ_{em} = 525 nm) and were obtained from Polysciences, Inc., Warrington, PA.

Instrumentation

The instrumentation consisted of an argon ion laser operated at 488 nm (Model 2001SL, Cyonics/Uniphase, San Jose, CA), PMT (R943-02, Hamamatsu, Middlesex, NJ) and housing (Thorn EMI, Inc., Fairfield, NJ), chopper (Model 9479, EG&G Ortec, Oak Ridge, TN), various lenses and optical filters (Corion, Holliston, MA), a 25 mm diameter mirror with a 2 mm hole bored through its center (prepared in house), monochromator (Model H-10, Instrument SA, Metuchen, NJ), strip chart recorder (Cole-Parmer Instruments Co., Chicago, IL), PMT-HV power supply (Model 556, EG&G Ortec, Oak Ridge, TN,), and picoammeter (Model 485, Keithley, Cleveland, OH). The arrangement of these components is shown in Figure 1 and is similar to a previously described arrangement.[6]

Construction

Construction of the MRB (see Figure 2) is begun by affixing six short sections of 200 μm i.d. capillary column and the fiber optic in a template such that they protrude through the template approximately 1/2" to 3/4". A reasonably symmetrical tip is formed

by smoothing the applied epoxy along the columns, thereby forming a bundle with the periphery defined by the columns (see Figure 2). As the epoxy hardens, the bundle is drawn out of the template slightly and more epoxy added in the same manner. By doing this, the tip is sufficiently long to allow grinding and polishing without loosing the symmetrical arrangement. To ensure the bundle fits tightly in the stainless steel frit shown in the figure, the template is constructed with the hole arrangement slightly smaller than the frit opening. Once the epoxy has set, the bundle is removed from the template and ground to a flat polished surface using a grinding wheel with fine lapping film. During the grinding process, the tip is sized using the frit. To keep the columns from becoming plugged with debris during the grinding, water is pumped through the columns.

To facilitate operation of the MRB, different diameter capillary columns are connected to the 200 µm columns just beyond the epoxy; two 520 µm columns are used as outlets, three 320 µm columns are used as inlets (two are used as rinsing inlets and one is used for beads introduction), and one of the original 200 µm columns is used to aspirate a given volume of sample (see below). Outlet and inlet columns are secured in 16 gauge needles with epoxy and terminated with a syringe on/off valve. The capillary column for bead injection is secured in a short length of tubing and, using a suitable ferrule and fitting, attached to the HPLC injection valve. Column lengths are arbitrary except for the aspiration column, which has a length dictated by the desired aspiration volume and is attached to an aspiration "T". Construction of the MRB is completed by affixing the hollowed frit on the polished capillary column/fiber optic bundle

such that a small volume (approximately 1 µl) sensing chamber is formed (see Figure 2). The operation of the MRB for each of the main evaluation steps is described below.

Sensor Conditioning Procedure

Before using the MRB, all leads were connected to their respective reservoirs and filled with water to remove any trapped air. It is important that air be removed to avoid problems with "signal altering voids" in the system. Water was also forced through the frit to remove air trapped in the pores. To ensure there were no leaks, water was aspirated through the frit and any air bubbles observed in the capillaries were an indication of a leak in the system. Throughout this study reagent *delivery* and *rinsing* steps are performed with the outlet columns sealed using the syringe on/off valves, thereby restricting flow through the frit, and *flushing* steps are performed with the outlets opened.

Reagent phase delivery

The first evaluation of the MRB involved determining the repeatability of delivering reagents to the sensing chamber. The ability to rinse and then flush the chamber was also evaluated. In this study, bead slurry and liquid reagent solutions are individually placed in the sensing chamber via their respective delivery capillaries. The Fluoresbrite beads (50 µl, 8 mg ml^{-1}) are introduced from the HPLC injection valve into the chamber. Once a stable fluorescence signal is obtained and recorded, the chamber is rinsed with approximately 25 µl of solvent. When performing actual assays, rinsing is necessary to remove unreacted material and possible interferents. After the rinse is completed, a second signal is

recorded, the tip is then cleared by flushing, and the process repeated. The repeatability of delivering liquid reagents was evaluated similarly. The FITC solution ($1x10^{-4}$ M) used in the evaluation is introduced from a reservoir and not as an aspirated sample (see below). This operation mimics the introduction, and subsequent removal of excess secondary reagents, such as labeled second antibody in an MRB-implemented sandwich assay.

Sampling by Aspiration

Samples are collected by connecting a 30 cm length of one of the 200 μm capillary columns (volume of approximately 10 μl) to the "T" configuration shown in Figure 2 and then aspirating with a 5 ml syringe while the sensing tip is in the sample. Once sample had been drawn past the junction in the "T", the tip is rinsed and a background signal recorded. The sample is then delivered via the aspiration lead from the junction in the "T". This operation reduces variations in the sample volume resulting from experimental variables such as frit permeability. The sample and rinse (approximately 50 μl) are collected in 1 ml volumetric vials and diluted to the mark prior to spectrophotometric measurement using a UV-Vis Diode Array Spectrophotometer (Hewlett-Packard, HP 8452, Palo Alto, CA). By comparing the absorbances with a calibration curve, sampled volumes are determined.

Combined Affinity Steps

After demonstrating the feasibility and determining the repeatability of performing isolated affinity steps, the steps were combined to measure fluorescently-labeled anti-rab-IgG and a possible interferent, human-IgG-FITC. In this study, the injection loop is

filled with immunobeads (10 mg ml^{-1}), sample collected, excess sample is rinsed from the sensing chamber, and the immunobeads are delivered to the sensing chamber. Once the beads are in place, a syringe pump is used to slowly deliver (flow rate approximately10 µl min^{-1}) aspirated sample, then rinse solution, to the immunobeads in the sensing chamber. At several points during the rinsing step, flow is stopped and the signal from the immunobead-bound anti-rab-IgG-FITC is recorded. The beads are then flushed from the chamber and the background signal recorded. This procedure is also used to obtain a dose-response curve for anti-rab-IgG-FITC. In this and other studies, the incident radiation is chopped (duty cycle about 6%) to avoid photodecomposition of reagents.

Results and Discussion

Fluoroimmunoassays (FIAs) are used extensively in clinical analysis.[28] The appropriate FIA protocol for a given analysis depends largely on the nature of the analyte. Natural fluorophors can be measured using a direct assay procedure that does not involve the complication of labeling reagents with fluorophors. We have developed "one-measurement" FOCS that are capable of remote direct FIAs[4,6,23] and recently demonstrated the feasibility of using the MRB to measure a small, fluorescent, carcinogenic compound.[25] The determination of nonfluorescent analytes is accomplished by either competitive-binding or sandwich assay techniques.[3] The former can be used for small analytes and involves competition between labeled analyte, from a standard source, and an unlabeled analyte, from the sample, for a limited amount of antibody. The antibody is usually immobilized on a support to facilitate removal of

excess reagents. Dose-response curves have a negative slope and exhibit short dynamic ranges. Labeled and unlabeled materials are generally mixed simultaneously, a process that does not easily lend itself to *in situ* analysis. Nevertheless, we used an immunochemical-based FOCS to perform remote, "one-measurement", competitive-binding assays.[17] Sandwich assays are generally performed using solid phase antibody (immobilized on a solid support) that binds the analyte, and a second antibody that is fluorescently-labeled and "tags" the analyte for measurement. Dose-response curves have a positive slope and a moderate dynamic range. However, the analyte must be fairly large ($M_r > 10^3$) so that the two antibodies can recognize different epitopes on the analyte.[3] Since the labeled reagent can be added subsequent to the initial incubation, the sandwich assay procedure is more easily adapted to continuous sensing. We are currently exploring the use of the MRB for performing sandwich-type FIAs.

The analytical significance of the MRB can be seen by comparing its operation and characteristics to that of one of the most popular instruments for performing FIAs. In the early 1980's, Pandex Corporation developed a versatile method of using immunobeads to perform FIAs.[29] With the Pandex method, the immunobeads are mixed with sample and various reagents and placed into funnel shaped microtiter wells that contain a frit at the bottom. The reagents and rinse solutions are drawn through the frit and the signal emanating from the trapped immunobeads is measured by front surface fluorimetry. The microtiter wells are arranged on a plate that permits many samples to be analyzed in a short period of

time. It is very significant that operational protocols for the MRB resemble those of the Pandex instrument. The addition of immunobeads, secondary reagents, and rinse solutions can be executed in various orders with the MRB to perform all of the aforementioned FIAs. While large numbers of samples can not be analyzed, repetitive measurements can be performed remotely in very small volume samples. The analytical attributes afforded by FIAs (e.g., high sensitivity and selectivity) are all potentially available with the MRB. The purpose of the work presented herein is to demonstrate that the steps common to FIAs can be performed individually and in combination in a repeatable fashion with the MRB, thereby illustrating its versatility as an analytical tool. Detectability, calibration capability, and the selectivity afforded by the specificity of immune reactions is also demonstrated. Future reports will emphasize the utilization of the MRB for specific assays.

Isolated Affinity Assay Steps

The performance of FIAs with the MRB requires that [1] controlled and adjustable amounts of immunobeads can be delivered to the sensing chamber, [2] secondary reagents can be reproducibly delivered to the chamber, [3] the chamber can be rinsed to remove excess liquid phase reagents and impurities that do not bind to the immunobeads, [4] sample solutions can be accurately and precisely aspirated, then delivered to the immunobeads, and [5] the contents of the chamber can be flushed completely so that the process can be repeated.

The results of an evaluation of these isolated affinity assay steps is presented in Table I. Fluorescently-labeled beads were delivered

to the chamber five times with a CV of 7.7 (first column in the table). Following each delivery, the beads were rinsed without being removed from the chamber (third column) and then flushed from the sensor. The complete flushing of the beads sometimes requires some aspiration to remove beads lodged in the corners of the sensing chamber. This ability to deliver solutions to the beads without removing them from the field of view of the fiber optic is critical to the operation of the MRB. To improve precision in an actual assay, the beads can be labeled with a spectrally distinct fluorophor to provide some normalization for the amount of beads introduced. The second column in the table illustrates the result of delivering an FITC solution to the chamber five times. The repeatability of this operation was very good (CV of 0.6) and, as the fourth column indicates, the FITC solution was efficiently removed from the chamber by rinsing.

The collection of a reproducible volume of sample is also critical to the operation of the MRB, particularly since there is no convenient means of normalizing for the volume of sample that is taken *in situ*. The aspiration function of the MRB is rather unique in that most previous sensors collect analyte via slow passive diffusion through permeable materials. In contrast, aspiration of analyte with the MRB is rapid and does not depend on analyte diffusivity or the permeability of a membrane or frit, which would likely change with time when real matrices are investigated. In fact, the capability of "cleaning-out" the MRB frit *in situ* by rinsing represents a significant advantage of this sensor.

The last three columns of Table I illustrate the repeatability of aspirating small and large analytes in water (series 1 and 2) and in blood serum (series 3). In all cases, the CVs for five aspirations were less than 8.0 with the biological matrix exhibiting the lowest CV. The differences in sampled volumes and the CVs are most likely due to the size and adhesion characteristics of the IgG, as compared to the FITC, and the viscosity of the matrix (the blood serum's higher viscosity would allow less diffusion from the aspiration column into other capillary columns).

Combined Assay Steps (Measurement of Anti-Rab-IgG-FITC)

Having verified that the MRB can accomplish commonly encountered steps in performing FIAs, experiments were conducted to demonstrate that the operations can be combined to conduct an actual assay. In this experiment, rab-IgG was covalently bound to silica beads, using a 1,1'-carbonyldiimidazole linkage that has shown to bind about 12 mg of IgG per gram of beads,[27] and anti-rab-IgG was measured. Although not studied in this work, proteins are normally measured using either a competitive-binding or sandwich assay procedure. Alternately, IgG can be measured using the protein's native fluorescence, however, the quantum efficiency is low and the maximum excitation wavelength (approximately 280 nm) is not convenient. Thus, we chose to use FITC-labeled antibody as the analyte ([anti-rab-IgG-FITC] approximately 0.1 mg ml^{-1}) in this measurement, which represents the case of the direct assay of a large molecule. We also chose a similar protein ([human-IgG-FITC] approximately 0.1 mg ml^{-1}) as an interferent. The advantages of employing this system include [1] availability and low cost, [2]

compatibility with the argon ion laser source, [3] established immobilization procedure,[27] and [4] the availability of structurally similar potential interferents (see below). Moreover, the sensing of IgG is clinically significant[30] and the measurement of a large protein with a FOCS is novel and demonstrates the aforementioned advantages of sampling by aspiration.

The procedure described in the Experimental Section was used to obtain the data in Table II. The signal levels shown in the table represent three points in the analysis and are recorded for the test system and two immunologically nonspecific systems. The three measurements are [1] the background level, [2] the signal after the aspiration step, and [3] the signal after immunobeads, sample, and rinse solution, respectively, are delivered to the chamber. Recall that the aspiration first fills (or partly fills depending on flow patterns) the sensing chamber then fills the aspiration capillary. As the immunobeads are delivered, the sample that occupies the sensing chamber is expelled and the signal drops to the background level. When the aspirated sample is delivered to the chamber, the signal increases dramatically then drops during rinsing as the excess sample analyte is removed from the chamber. The change in signal during operation is described later with reference to Figure 3. Table II provides encouraging data, as well as an indication that refinements in the sensor chamber configuration and optimization of operating procedures are necessary.

An important point illustrated in Table II is that the repeatability is actually better for the combined affinity steps than for the isolated aspiration step, alone (the CV for immune specific assay is 6.2). This

indicates that the combined variance in a particular assay is not necessarily an additive function of those for the isolated steps. This is not surprising since the evaluation of the isolated steps does not perfectly mimic the steps in every assay. In particular, the effects of differences in the amount of anti-rab-IgG-FITC solution aspirated in the present study are reduced by the fact that, as the sampled volume is passed through the immunobeads, the most readily observed beads are first saturated with the analyte and, subsequently, beads that are less easily viewed by the fiber optic are involved in the immune reaction. Nevertheless, the wide variety of available assay protocols validates the previous evaluation of repeatability of the isolated assay steps, as it can provide some insight into the expected assay precision.

A second notable advantage of the MRB in this assay is the selectivity afforded by the specificity of the immune reaction. After rinsing, the anti-rab-IgG-FITC signal is appreciable, while the assay of similar protein (human-IgG-FITC) is statistically negligible. This indicates that the immunobeads can be rinsed to remove nonspecific interferents with retention of analyte. The blank signal for anti-rab-IgG-FITC, obtained using nonaffinity beads, is also negligible. This excellent selectivity is particularly important in sensing applications since, unlike conventional analyses, benchtop isolation of analyte via extractions, chromatographic separations, etc. is difficult or impossible. Hence, samples are inherently complex and analyses are susceptable to interference.

Figure 3 readily illustrates several points which are not apparent from the table. The first is the signal obtained from the sampled

anti-rab-IgG-FITC when initially delivered to the sensing chamber that is filled with immunobeads is higher than the aspiration signal. This is probably due to the reaction and concentration of the analyte on the beads closest to the fiber before the beads redistribute during the rinse (see below). The nonspecific human-IgG-FITC and the nonaffinity beads do not exhibit this signal pattern. Variation in the initial heights of the aspiration signals for the two types of IgGs are due to the variation in the labeling ratios.

The second point is the decrease in the signal as the rinsing continues. This occurs despite the fact that the amount of immunobead-bound antibody in the sensing chamber is several times the amount of anti-rab-IgG-FITC in the 10µl sample. This decrease is associated with [1] removal of unbound protein, [2] removal of the specifically bound protein, and [3] redistribution of the beads. Having removed the unbound protein in the first ten minutes of the rinse (see Figure 3), removal of specifically bound protein does not seem to be a major contributor to the signal decrease. This can be seen in the figure as the rinse continues for an additional ten minutes with negligible signal reduction. The decrease in signal due to immobead redistribution is most problematic and probably occurs when the rinsing mixes the reacted and unreacted beads and pushes the beads to the outside walls of the sensing chamber, out of the fiber optic field of view. Movement out of the field of view is due to the walls of the frit being thinner than the end (see Figure 2). This is supported by the observation that during rinsing, the flow is most prominent through the walls and not the end of the frit. Studies with a new frit design having approximately

equal thicknesses for the end and the walls are in progress. We are also currently working on a delivery system consisting of three inlets and three outlets (symmetrically arranged), through which all reagents will be introduced and removed. It is hoped that this arrangement will permit more "symmetrical" flow patterns within the sensing chamber; thereby minimizing the effects of redistribution. Other operational and design parameters which were not optimized in these preliminary experiments are bead concentration, reagent phase flow rates, chamber geometry, and frit permeability.

A dose response curve was constructed over a range of 0.011 mg ml^{-1} to 0.11 mg ml^{-1} anti-rabbit IgG-FITC (see Figure 4). Excluding the highest concentration data point, the correlation coefficient for the linear portion of the curve was 0.9969. The nonlinear portion of the curve is possibly due to the saturation of those beads within the field of view before rinsing removes the sample and redistributes the beads. The LOD (S/N = 2) is approximately 8×10^{-3} mg ml^{-1} (5×10^{-8} M) but could possibly be improved by increasing the volume of aspirated sample. By increasing the sample volume, the sample would come in contact with the beads for a longer time before rinsing and redistribution influences the signal. The absolute LOD is approximately 8×10^{-5} mg (5×10^{-13} moles). Though the linear dynamic range was limited for this MRB, it is not believed that this is an inherent difficulty with the design but rather due to the fact that the operational and design parameters have yet to be fully developed. Both the dynamic range and LOD are expected to improve when the changes discussed above are implemented.

Future work will be necessary to realize the full potential of this system for performing long-term FIA-sensing in real matrices using sandwich, competitive-binding, enzyme, etc. assay protocols.

Acknowledgements

This research was supported by the National Science Foundation under contract number CHE-8708581 and the Division of Chemical Sciences, Office of Basic Energy Research, U.S. Department of Energy under contract number DE-FG05-86ER13613 with the University of Tennessee, Knoxville.

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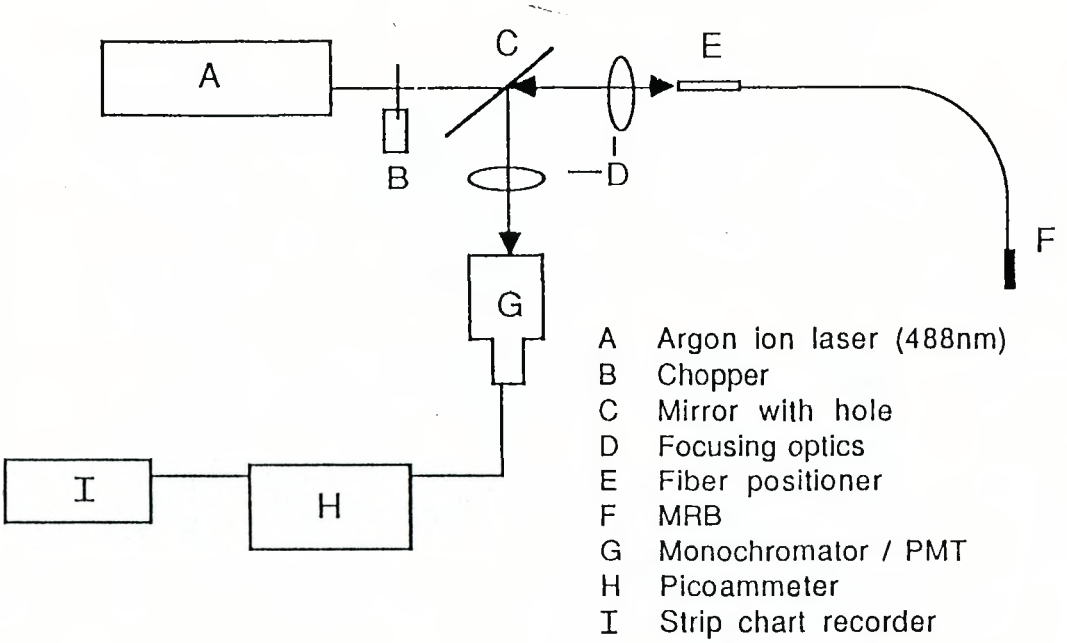
LIST OF FIGURE CAPTIONS

Figure 1: Block diagram of the optical configuration used with MRB.

Figure 2: Diagram of frit and column configuration at MRB sensing chamber (A) and reagent delivery system (B).

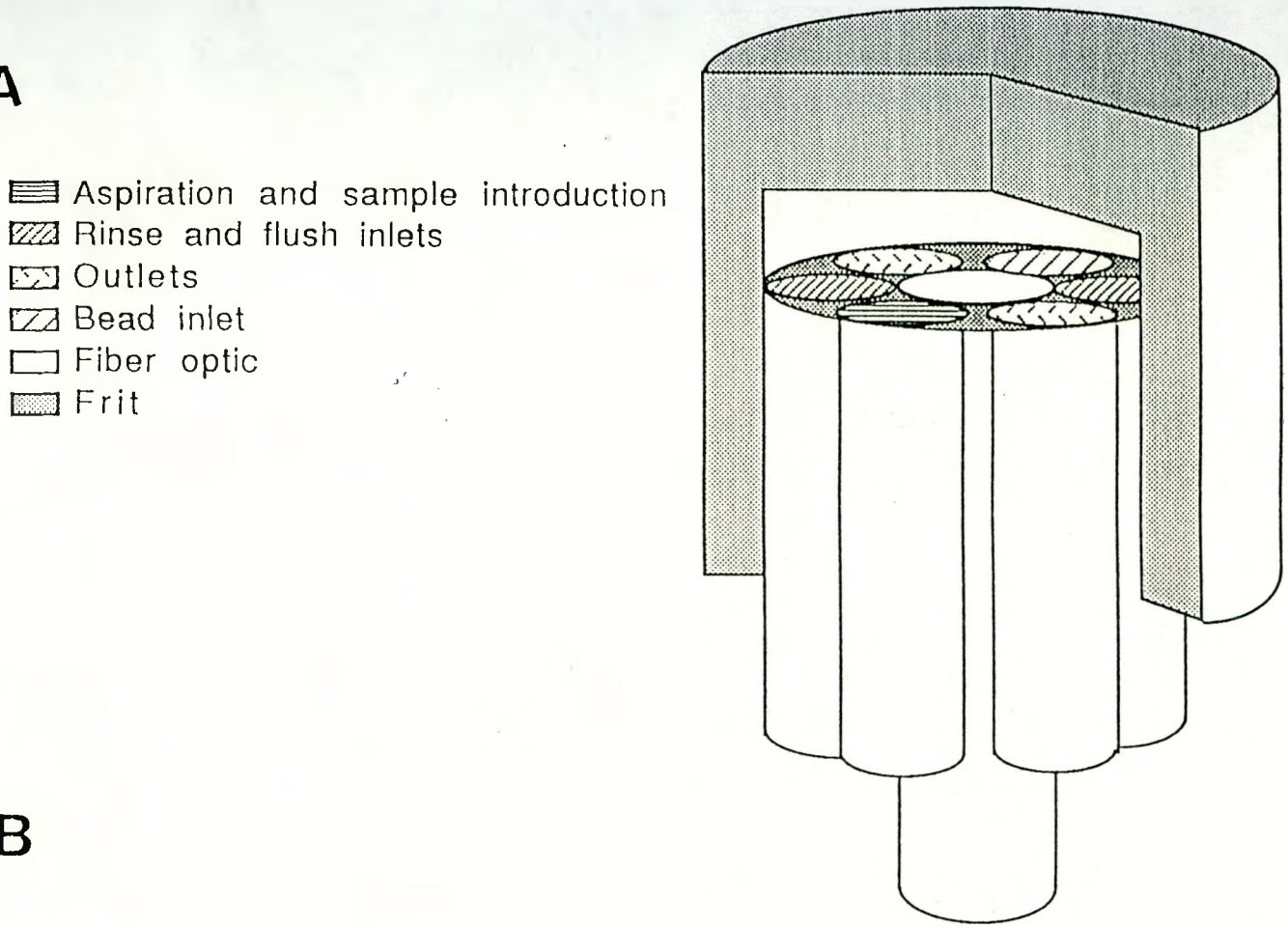
Figure 3: Temporal representation of the signals obtained in the assay of anti-rab-IgG-FITC (see Table II for further information).

Figure 4: Dose-response (i.e. calibration) curve for anti-rabbit IgG-FITC assay.

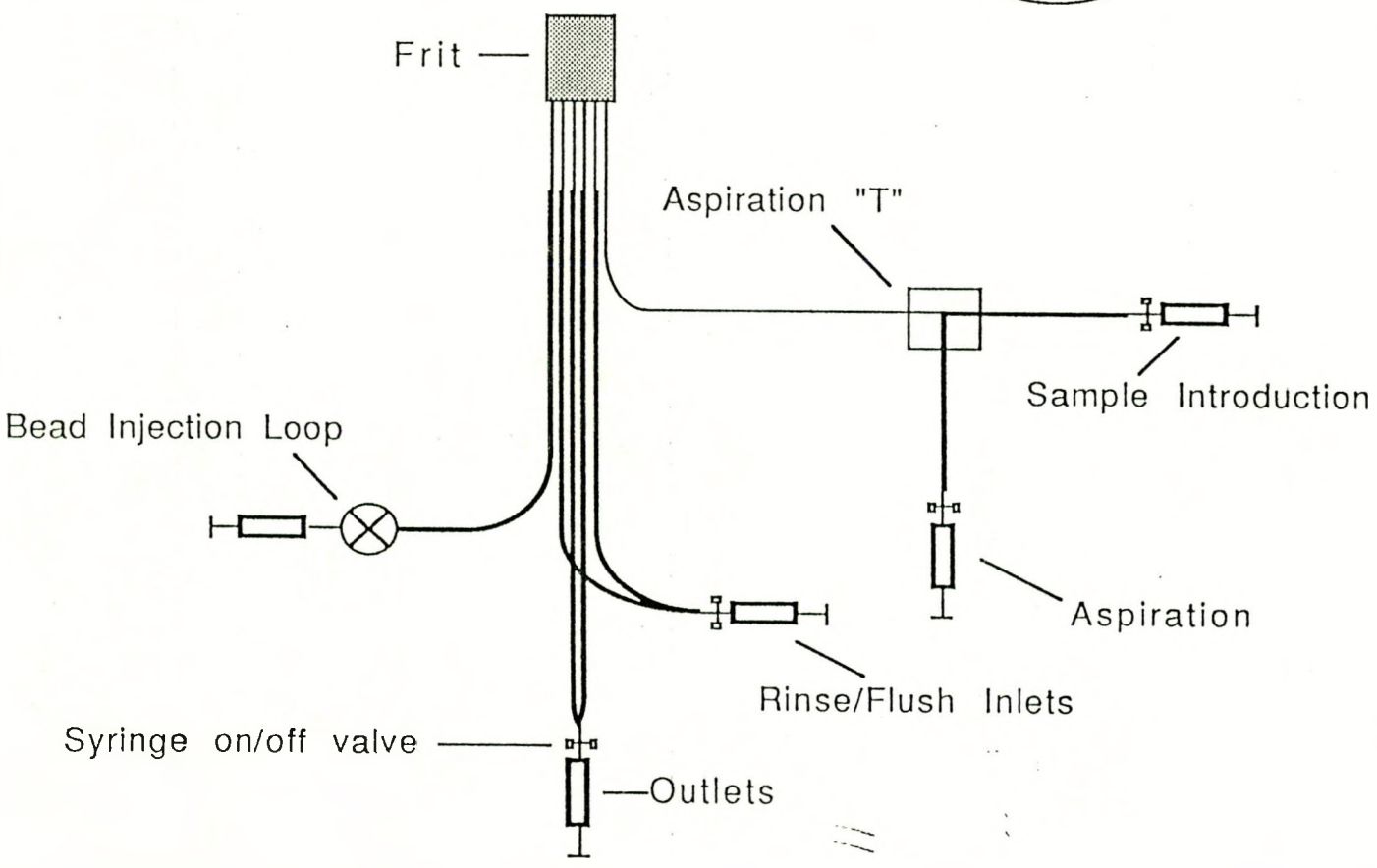


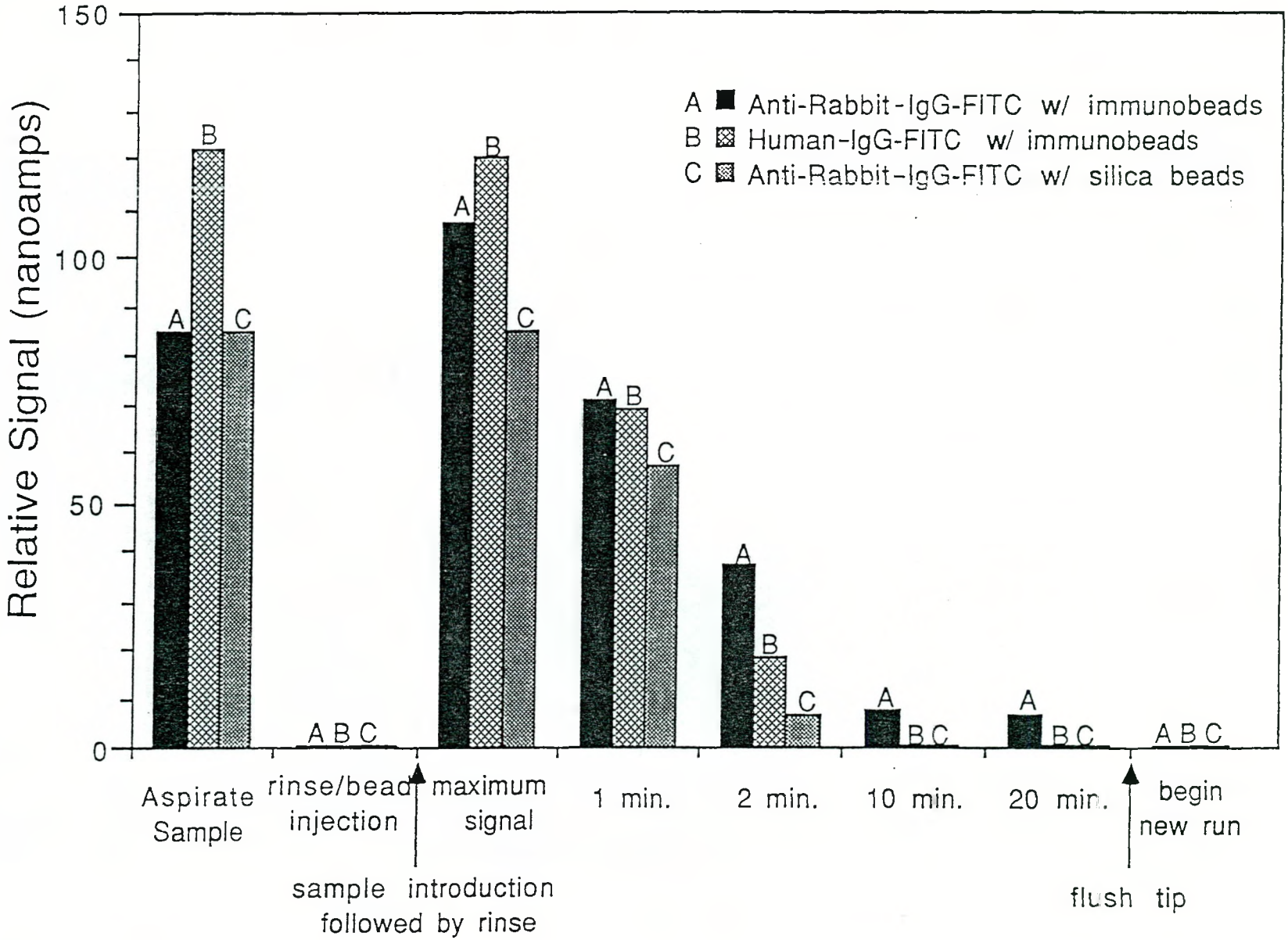
A Argon ion laser (488nm)
B Chopper
C Mirror with hole
D Focusing optics
E Fiber positioner
F MRB
G Monochromator / PMT
H Picoammeter
I Strip chart recorder

A


Aspiration and sample introduction
Rinse and flush inlets
Outlets
Bead inlet
Fiber optic
Frit

B


Frit
Aspiration "T"
Sample Introduction
Bead Injection Loop
Aspiration
Rinse/Flush Inlets
Syringe on/off valve
Outlets


150
100
50
0
Relative Signal (nanoamps)
A Anti-Rabbit-IgG-FITC w/ immunobeads
B Human-IgG-FITC w/ immunobeads
C Anti-Rabbit-IgG-FITC w/ silica beads
A B C
Aspirate Sample
rinse/bead injection
maximum signal
1 min.
2 min.
10 min.
20 min.
begin new run
sample introduction followed by rinse
flush tip

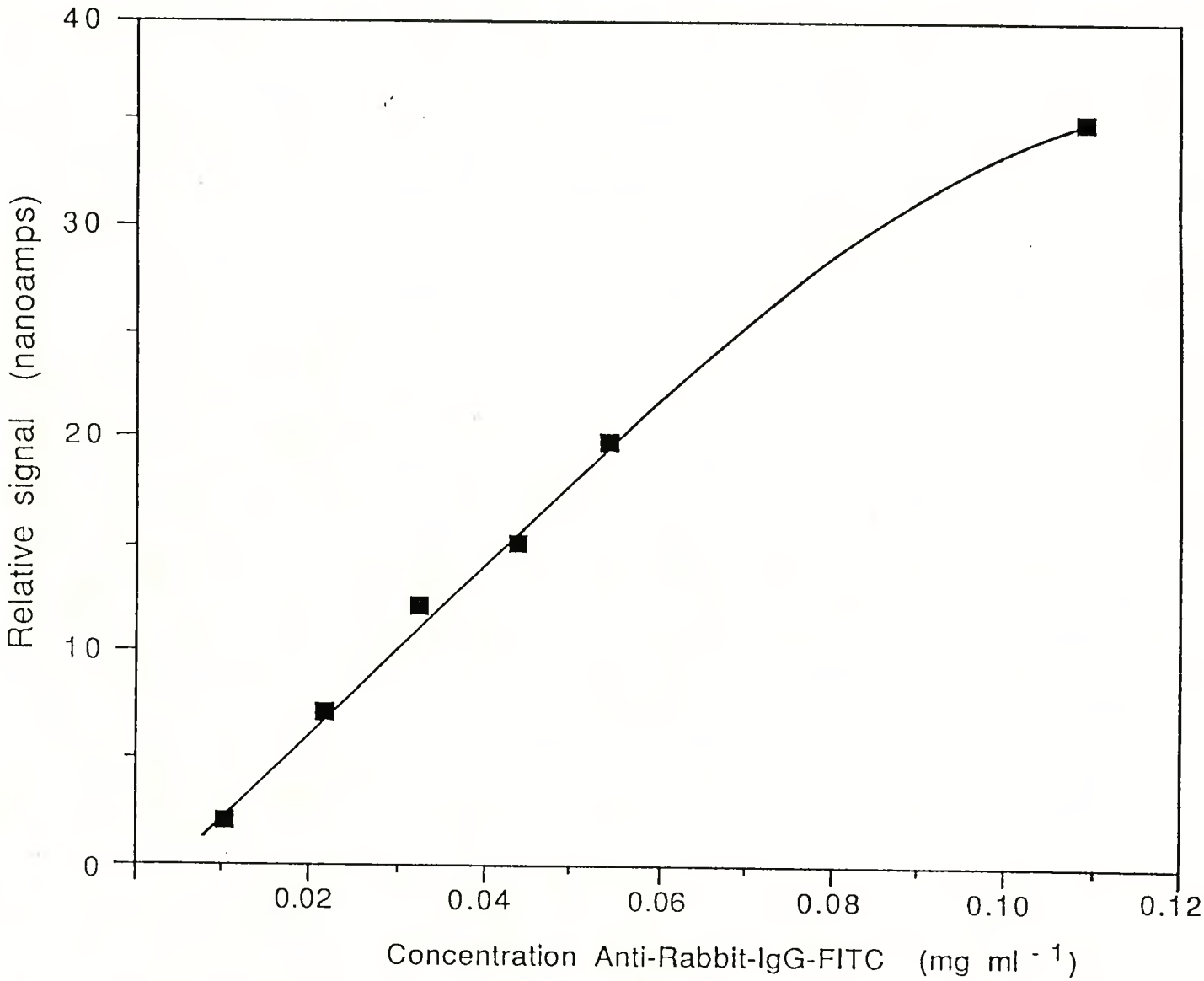

40
30
20
10
0
Relative signal (nanoamps)
0.02
0.04
0.06
0.08
0.10
0.12
Concentration Anti-Rabbit-IgG-FITC (mg ml - 1)

TABLE I. Signal and Reproducibility Data for Performing Isolated Affinity Assay Steps With the MRB

Measurement	Delivery of Reagents (signal,nA)		Rinsing of Reagents (Signal,nA)[c]		Aspiration Volume (μl)[d]		
	Solid Phase[a]	Liquid Phase[b]	Solid Phase	Liquid Phase	Series 1	Series 2	Series 3
0	-	-	-	3.78	-	-	-
1	510	25.2	510	3.70	12.3	10.5	13.4
2	560	25.2	560	3.83	13.4	12.9	13.4
3	470	25.3	470	3.78	11.7	11.5	13.9
4	550	25.2	550	3.73	11.9	11.4	13.2
5	470	25.3	470	3.78	12.1	11.4	13.0
X	510	25.3	510	3.77	12.0	11.9	13.0
CV	7.7	0.6	7.7	1.2	5.4	7.5	2.5

Notes: See Experimental Section for further details

(a) 50 μl of 8 mg ml^{-1}, 6 μm, Fluoroesbrite beads delivered to sensing chamber

(b) 20 μl of 10^{-4} M FITC delivered to sensing chamber via inlet capillary columns with unlabeled beads in chamber

(c) 20-30 μl of water delivered to sensing chamber via inlet capillary columns (bead signal unchanged, FITC expelled). Between measurements, 50-60 μl of water delivered to sensing chamber with outlet capillaries open to flush the sensor

(d) Aspiration of 10^{-4} M FITC (series 1); 5 mg ml^{-1} rab-IgG-FITC, FITC concentration = 1.4×10^{-4} M (series 2); and 5 mg ml^{-1} rab-IgG-FITC in human blood serum (series 3)

TABLE II. Demonstration of Combined Affinity Assay Steps With the MRB

Measurement	Signal (nA) [Background/Aspiration [a]/Post Delivery [b]]		
	Rabbit-IgG affinity beads		Non-affinity beads
	Anti-rabbit-IgG-FITC	Human-IgG-FITC	Anti-rabbit-IgG-FITC
1	13 / 90 / 21	16 / 140 / 16	15 / 100 / 16
2	14 / 91 / 23	15 / 138 / 16	14 / 98 / 15
3	13 / 92 / 20	15 / 138 / 15	14 / 100 / 14
4	13 / 96 / 20	15 / 136 / 15	14 / 98 / 14
5	12 / 100 / 22	16 / 138 / 17	14 / 96 / 14
$\bar{X}$	13 / 94 / 22	15 / 138 / 16	14 / 98 / 15
CV	5.4 / 4.3/ 6.2	3.6 / 1.0/5.3	3.1/ 1.7/ 6.1

Notes: See Experimental Section for further details

(a) Signal measured using MRB following aspiration (approximately 10 μl) and before delivering beads to the sensing chamber

(b) Signal measured after delivering [1] beads, [2] aspirated sample, and [3] rinse solution to sensing chamber.

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