

# Utilization of Bradford Assay to Aid in Development of a Novel Point-of-Use Immunobiosensor

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## Abstract

Immunodiagnosics play a critical role in disease diagnosis and monitoring. Further advancement of immunoassays will extend the reach of medical technology to allow testing in the field with inexpensive and disposable point-of-use biosensors that respond to target molecule(s) (antigens) in test solutions by a change in one or more of the biosensor's properties. An immunobiosensor that utilizes a biochemically-enhanced paper test strip is being researched and developed in our laboratory. The work presented here does not focus on the design or use of the immunobiosensor, but rather on development of an analytical tool/assay method to better understand the immunobiosensor's test strip composition during its manufacturing process and its functional behavior during an assay. The objective of the work described here was to develop and apply a modified Bradford assay to enable determination of how much antibody is crosslinked to the paper strip during manufacture via measurement of the depletion of the applied antibody from the applied solution. Additionally, this modified Bradford assay allowed for the quantification of immobilized-antibody capture (specific binding) of antigen in an "active test strip" during testing, and for the quantification of nonspecifically bound antigen in a "reference test strip" during testing to enable determination of the antibody's specific antigen-binding activity (and percent loss of activity). This method was also useful for determining rinse times at various steps during test strip manufacturing required to remove free, unbound protein that is present in the pores of the paper strip and for determining exposure times needed for various solution/protein treatments. In our study it was found that more than 80% of the applied antibody was bound to the paper strip during the crosslinking step with different antibodies experiencing similar crosslinking efficiencies. The activity of the crosslinked antibody in the test strip was also quite good, retaining 22% of the supplier's reported antibody's specific antigen-binding capacity, much greater than typically found in ELISA.

## Introduction

Medical diagnostics has revolutionized modern medicine, but drawbacks and limitations are still present. Most medical service providers, such as hospitals and doctor offices in developed countries, currently outsource their laboratory testing to central laboratories. These central laboratories then perform the desired moderate- or high-complexity tests. These central laboratories will report the test results back to the physicians for diagnosis and potential treatment. For low-complexity, time-sensitive tests the medical service providers might maintain a small laboratory on their premises.<sup>1</sup>

There are some issues with this type of healthcare system. Most developing countries do not have the clinical infrastructure to support widespread medical laboratories yet have the majority of world's population impacted by disease.<sup>2</sup> Transportation of the collected specimens requires temperature control which can add unnecessary risk along with extended diagnosis times. Several diagnostic tools involving polymerase chain reaction, mass spectrometry, and fluorescence-activated cell sorting are very time consuming, costly, and have a shortage of experienced technicians.<sup>3</sup> These are some aspects of current diagnostic testing that limit their applications and can result in avoidable patient mortality.

Point-of-care tests (POCT) aim to be a simple, cost-effective, and low-complexity solution to increasing demands on medical care. POCT are defined as any diagnostic test administered at or near the location of the patient.<sup>4</sup> The benefits of these devices are the lower amount of training, minimal reagent requirement and faster result turnaround compared to standard laboratory testing.

Within the emergency department (ED) POCT can provide faster discharge rates, shorter lengths of stay, and increased patient throughput.<sup>4</sup> There are delays in administration of treatment and medications when the ED becomes overcrowded.<sup>5,6</sup> With faster test results, POCT could have beneficial effects on ED responses to potential severe sepsis,<sup>7</sup> venous thromboembolic disease,<sup>8,9</sup> and acute coronary syndrome.<sup>10,11</sup> POCT can enable early detection of cancer biomarkers such as prostate specific antigen,<sup>12</sup> carcinogenic-embryonic antigen,<sup>13</sup> CA15-3 antigen, her-2/neu,<sup>14,15</sup> Ca19-9,<sup>16</sup> and CA125,<sup>17</sup> to dramatically improve treatment outcomes. Additionally, POCT can provide consumers with in-home screening of biomarkers to alert the user to consult a physician. Other applications for POCT would be veterinary medicine, space missions, sports medicine, traumatic brain injuries, and surgeries.<sup>18</sup>

Immunoassays/biosensors quantify the concentration of certain proteins (antigens) in test samples by their interaction with test-incorporated specific antibodies. These immunoassays/biosensors are designed with two major components: a bioreceptor (antibody) that captures the antigen from an applied sample and a transducer which converts the antigen-antibody binding into a detectable signal. The enzyme-linked immunosorbent assay (ELISA)<sup>19</sup> is currently the workhorse in research laboratories for these assays.<sup>3</sup> However, the high cost and complexity of ELISA methods limit their applications. New generations of immunoassays are being investigated, but still possess restrictions.<sup>20-23</sup>

To fulfill the need for stable, reliable, and simple point-of-care diagnostic techniques,<sup>2</sup> many laboratories and companies are developing POCT. In our laboratory an immunobiosensor (test strip), utilizing a chemically modified paper strip with crosslinked immunoglobulin G molecules, is being developed and studied.

During test strip manufacturing, it is essential for the active and reference test strips to receive the same manufacturing processing, conditions, and treatment. The active test strip- and reference test strip-incorporated antibodies are different, but the amount of antibody incorporated into each test strip must be the same. If there is any significant difference in the amount of strip-incorporated antibody between test strips (reference and active), the interpretation of the transducer response would be compromised. To address this issue, the active and reference antibody solutions used to coat the test strips are adjusted to have equivalent antibody concentrations (using  $A_{280\text{nm}}$  spectrometry measurements). In addition to controlling the antibody concentrations, the same volume of antibody solution is applied to the paper strips during the crosslinking step of manufacturing. To quantify this variability, a modified Bradford assay was developed and used to determine the mass of antibody immobilized in the active test strip and reference test strip.

The final stage of test strip manufacturing involves storage of the paper strips in a 20 mg/mL bovine serum albumin (BSA) solution with sodium azide. The paper strips remain in the storage solution at 4 °C for at least 24 hours (preferably longer) prior to use. The BSA in the storage solution is used to adsorb to all non-protein covered surfaces and minimize nonspecific binding of protein to the test strip during antigen testing at a later date. Protein within the test strip greatly affects the transducer response during test strip testing/analysis. After exposure to an antigen-containing solution (testing), the test strip is washed to remove as much free/loosely bound protein as possible in a repeatable fashion. The duration of washing required for the majority removal of loosely bound protein was determined by analyzing the amount of free protein remaining in the test strips washed for varying amounts of time using the modified Bradford assay described in this report.

When testing a sample containing the antigen specific for the active test strip, the active test strip response is dependent on the amount of antigen that binds specifically and nonspecifically (irreversible physical adsorption) to the test strip. Reference test strips are manufactured using the same process that is used for the active test strips, but with an antibody specific for a different antigen that is not present in any test samples. The reference test strip will only experience nonspecific binding with zero specific binding. The difference between the response of the active and reference test strips is due to specific binding and can be related to the concentration of antigen in the test solution. To understand how much antigen was specifically bound to the test strip for a particular test sample, a modified Bradford assay was developed for our study and used to evaluate the quantity of depleted antigen from the applied test solution for both the active and reference test strips. This was done to evaluate what percentage of bound/immobilized antibody was still active after the manufacturing process.

## Methods

### *Materials*

The Bradford reagent (B6916), anti-rabbit IgG from goat (R2004), rabbit IgG (I5006), and bovine serum albumin (A3059) were purchased from Sigma-Aldrich. All materials were stored at 4 °C.

### *Overview of Test Strip Manufacturing*

The test strips are fabricated from paper (type not disclosed). A standard paper trimmer with reference blocks is used to cut the paper into multiple 5 cm by 0.5 cm rectangular strips. These paper strips are chemically modified to form a scaffold for the crosslinking of immunoglobulin G (IgG) molecules. Ultimately the paper strip scaffold, comprised of other undisclosed compounds, serves as the test strip's transducer while the crosslinked IgG represents the bioreceptor.

### *Bradford Assay Technique*

The Bradford assay is a protein determination method developed by Marion Bradford in 1976 that utilizes the binding of Coomassie Brilliant Blue G-250 to protein.<sup>24</sup> The dye actively binds to arginine and lysine residues on proteins.<sup>25</sup> The concentration of a protein in solution, with a detectable limit of 1 µg/mL, can be determined by comparing the absorption of the assay at a wavelength of 595 nm to a calibration curve.<sup>24</sup> There are some inherent drawbacks in the original design. One being that each protein contains a different amount of arginine and lysine residues. This causes different absorbance responses between different proteins but can be accounted for by developing separate calibration curves. There is also a nonlinearity in the assay's response to varying protein concentrations.<sup>24</sup>

To account for the nonlinearity of the original Bradford assay, Zor and Selinger (1996) proposed a modified technique that utilizes the ratio of 590-nm absorbance to the 450-nm absorbance.<sup>26</sup> This change measures the ratio of protein-bound dye to free dye in the solution, whereas the original Bradford assay only measures the concentration of dye bound to protein.

Additional minor modifications of the linearized Bradford assay technique were made for the utilization in this study. The proposed 1-mL assay from Zor and Selinger was used with the substitution of measuring the 595-nm wavelength absorbance instead of 590 nm.<sup>27</sup> Instead of mixing samples within the cuvette, the assay was prepared and incubated within one vial and then wholly transferred into a cuvette for analysis.<sup>27</sup> All assays were performed at room temperature with an incubation time of 32 minutes.

### *Quantification of Antibody Incorporation into the Test Strip*

Prior to antibody incubation, the activated paper-strip scaffold (method undisclosed) contains crosslinking reactive sites for IgG to covalently bind. To integrate the IgG antibody into the paper strip, the paper strip was carefully placed with tweezers onto a petri dish. Multiple paper strips were laid in parallel to each other and each petri dish could fit eight paper strips with

ample space in between. Two petri dishes were used for the active antibody treatment and two petri dishes were used for the reference antibody treatment. After the paper strips were placed, the petri dish was tilted and the excess liquid remaining on the paper strips from the previous stages was aspirated using a clean blunt syringe needle without direct contact to the paper strips. A micropipette was used to apply 0.1 mL of a 457  $\mu\text{g}$  antibody/mL solution, 0.01 M phosphate-buffered saline at 7.0 pH (7.0 PBS), to each paper strip in drops that spread across the surface of each paper strip. The petri dish was covered to reduce evaporation and left at room temperature for two hours.

After the two hours, the remaining solution on each paper strip was collected by using a micropipette. Direct contact with the test strip was avoided. All antibody-containing solutions remaining on the paper strips within a single petri dish were combined into one 1.8-mL vial. Three dilutions were made by combining 40  $\mu\text{L}$  of the petri dish sample with 160  $\mu\text{L}$  of 7.0 PBS. The modified Bradford assay method was used to analyze the dilutions. These two-hour samples were assumed to be at equilibrium with the paper strip (i.e. all possible reactive sites were reacted, or all antibody had been crosslinked to the paper strip), and no more crosslinking was occurring. The measured antibody concentration in solution represented the unbound antibody still in solution. The petri dish before paper strip placement, after paper strip placement, sample application, and sample removal was weighed. This information was used in subsequent calculations important to data analysis.

#### *Determination of Test Strip Washing Dynamics*

A washing apparatus was made with six 5-mL vials. Each vial had a blunt syringe needle that supplied 18 M $\Omega$  water from a peristaltic pump from a tubing manifold. The pump was set to flow 160 mL/min out of each syringe needle. One test strip was placed in each 5-mL vial while the pump was running for washing. The 18 M $\Omega$  water flowed into each vial and overflowed into waste.

Different test strip washing times of 2.5, 5, 7.5, 10, 15, and 20 minutes were examined. Six test strips were washed simultaneously. At 2.5 minutes, one syringe needle was removed from one 5-mL vial while the other five test strips continued to be washed. At 5 minutes another syringe needle was removed. This process was repeated until 20 minutes was reached, and the final syringe needle was removed from the vial.

When a syringe needle was removed from the vial, the remaining liquid and test strip was left for 20 minutes. Any protein that could desorb from the test strip would equilibrate with the surrounding liquid. After the 20-minute waiting period, a micropipette was used to mix the wash vial solution and take a 0.6 mL sample. For the 2.5- and 5-minute vials, three dilutions were made by combining 15.4  $\mu\text{L}$  of the wash-vial sample with 184.6  $\mu\text{L}$  of 18 M $\Omega$  water. Three 0.2-mL undiluted samples were prepared for all other washing times. All prepared samples were analyzed using our modified Bradford assay technique.

## *Quantifying the Extent of Antigen Specific Binding and Percent Activity of Immobilized Antibody*

There is potential of BSA desorbing from the test strip and entering the pure antigen-containing solution during studies involving determination of antigen specific binding. Additional BSA in the antigen solution, when analyzed with the Bradford assay, would appear as an abnormally high antigen concentration since the Bradford assay measures total protein content. To remove loosely adsorbed BSA, the test strips were washed for at least 20 minutes (the time determined from the washing study) to remove any free BSA before antigen testing. This ensured that all potential BSA interference was eliminated.

Three replicates, three washed active test strips and three reference test strips, were used for each analysis. The test strips were placed in a separate petri dish according to the antibody type (active or reference). The excess liquid was removed from the test strip by aspiration while tilting the petri dish. No contact was made between the micropipette tip and the test strip.

Both the active and reference test strips were exposed to 0.1 mL of 413  $\mu\text{g}$  antigen/mL in 7.0 PBS. The solution was applied in drops that spread across the exposed test strip surface while avoiding any physical contact with the test strip. The petri dish was covered to minimize evaporation.

The remaining antigen solution on the test strips of a single petri dish was pooled into a 1.8-mL vial after two hours. Direct contact with the test strip was avoided. Three dilutions were made by combining 40  $\mu\text{L}$  of a single petri-dish sample with 160  $\mu\text{L}$  of 7.0 PBS. These samples were analyzed using the modified Bradford assay technique. This provided the concentration of the antigen remaining in the solution after two hours of exposure.

### *Data Analysis*

Actual dilution factors were calculated for each modified Bradford assay sample. Any dilution factor outliers were identified using the modified Thompson tau technique for a single variable. Those identified as outliers (if any) were ignored in the data analysis. The mean, sample standard deviation, and 95% confidence intervals were determined for all collected data. Two-sided hypothesis tests were used to compare the means between the final active and reference antibody concentrations. A one-sided hypothesis test was used to compare antigen binding between the active and reference test strips. An alpha of 0.05 was used for all statistical analyses.

## **Results**

### *Efficiency of Antibody Crosslinking to Test Strips During Manufacture*

Fifteen active and 15 reference test strips were analyzed for the antibody crosslinking study. Eight active test strips were incubated in one petri dish while the other seven were in another petri dish. The same scheme was used for the reference test strips. The mean paper strip mass for both active and reference test strips after aspiration and before the antibody application was 0.1443 g per paper strip. Each paper strip received a mean of 0.1004 mL of the respective antibody solution—assuming a density of 1.0 g/mL for all liquids—at a concentration of 457  $\mu\text{g}$

antibody/mL (7.0 PBS), and its mean time-zero mass was 0.2448 g. After the two-hour incubation period, the mean mass of each paper strip and remaining liquid solution was 0.2366 g (evaporation loss of 0.0081 mL). Each test strip, after rinsing adequately with 18 MΩ water, had a mean mass of 0.0445 g when completely dry. Thus, the mean volume of liquid remaining in the paper strips was 0.1921 mL at the end of antibody incubation. Modified Bradford assays were performed in triplicate for the pooled liquid from each petri dish containing 7 or 8 paper strips each. The concentrations of antigen remaining in the pooled liquid solution after incubation are shown in Table 1.

*Table 1*  
Concentrations of Antibody Remaining in Solution After a Paper Strip Exposure Period of Two Hours.

Petri dish	Active Antibody Concentration (µg/mL)			Petri dish	Reference Antibody Concentration (µg/mL)		
	n	Mean (SD)	95% CI		n	Mean (SD)	95% CI
1	3	33.6 (2.6)	[30.7, 36.5]	3	3	41.6 (6.4)	[34.4, 48.8]
2	3	55.6 (8.1)	[46.4, 64.7]	4	3	36.7 (3.9)	[32.3, 41.2]
<b>Total</b>	<b>6</b>	<b>44.6 (13.2)</b>	<b>[34.0, 55.1]</b>	<b>Total</b>	<b>6</b>	<b>39.1 (5.4)</b>	<b>[34.8, 43.5]</b>

Note. n = number of assays; SD = standard deviation; CI = confidence interval,  $\alpha = 0.05$ .

The mean concentrations for the active and reference antibody remaining in solution at the end of incubation dropped to 44.6 (SD = 13.2) and 39.1 µg/mL (SD = 5.4), respectively. At 95% confidence there was not a difference in concentrations between the two reference-antibody petri dishes (two-sample t test assuming unequal variance,  $t(3) = 1.126$ ,  $p = 0.342$ ). However, there was some difference between the two petri dishes for the active antibody (two-sample t test assuming unequal variance,  $t(2) = -4.490$ ,  $p = 0.046$ ). The mean for all active antibody samples (44.6 µg/mL) and reference antibody samples (39.1 µg/mL) were found to be equivalent (two-sample t test assuming unequal variance,  $t(7) = 0.935$ ,  $p = 0.381$ ).

The concentrations from Table 1 represent the concentrations of antibody that did not bind to the paper strip. It is necessary to calculate the antibody mass remaining in solution to calculate the mass of antibody immobilized in the test strip. Two methods of analysis were used to find the mass of antibody bound to the paper strips.

Method A: There is a possibility that the antibody could not significantly penetrate the paper-strip scaffold. This barrier would prevent the mixing of applied antibody solution and water within the paper strip pores, reducing the working solution volume to 0.1004 mL (the applied antigen solution volume). In this case, using the final antibody concentrations, the amount of antibody crosslinked to the active and reference paper strips was 41.3 and 41.8 µg, respectively, using Method A Table 2.

Method B: If the antibody in the 0.1004 mL applied sample uniformly mixed with all liquid contained within the paper strip pores, the total liquid volume would be 0.1921 mL. Therefore, 8.6 µg of active antibody was not crosslinked to the paper strip, and 7.6 µg of reference antibody

was not crosslinked. With a starting antibody mass of 45.9  $\mu\text{g}$  in the 0.1004 mL sample, 37.1 and 38.1  $\mu\text{g}$  of antibody was crosslinked to the active and reference strips, respectively, using Method B Table 2.

*Table 2*  
Masses of Antibody Incorporated into Paper Strips.

Test Strip	Antibody Bound to Paper Strip ( $\mu\text{g}$ )	
	Mean - A	Mean - B
Active	41.3 $\pm$ 0.4	37.1 $\pm$ 0.4
Reference	41.8 $\pm$ 0.4	38.1 $\pm$ 0.4

Note. A = Method A; B = Method B

*Removal of Blocking BSA from Test Strips*

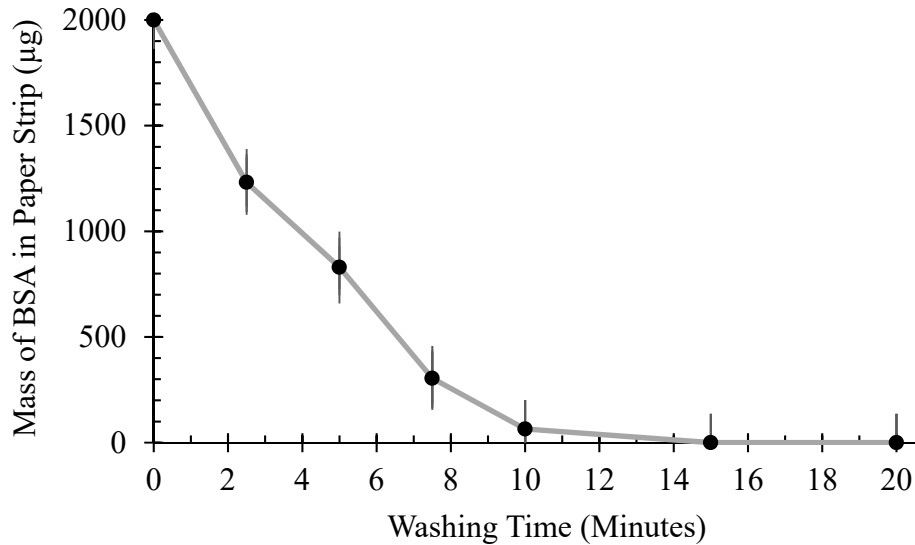
After washing the prepared test strips that had been stored in BSA solution for select periods of time, the amount of free BSA that remained in the test strip was determined by extracting the BSA into 5-mL of 18 M $\Omega$  water. The concentration of BSA in these solutions decreased as test strip wash times increased as seen in Table 3. The concentrations for test strips washed for 15 and 20 minutes were reported as negative. This is a consequence of the modified Bradford assay calibration curves as concentrations below the assay’s detectable limit (1  $\mu\text{g}/\text{mL}$ ) have an absorbance ratio that produces negative concentrations. The only possible interpretation of these concentrations is that they are between 0 and 1  $\mu\text{g}/\text{mL}$ .

*Table 3*  
Desorbed BSA Remaining in Test Strips Washed for Different Time Periods (Extracted into 5 mL of Water).

Time (min)	BSA Concentration ( $\mu\text{g}/\text{mL}$ )			Time (min)	BSA Concentration ( $\mu\text{g}/\text{mL}$ )		
	n	Mean (SD)	95% CI		n	Mean (SD)	95% CI
2.5	3	246.3 (3.9)	[241.9, 250.8]	10	3	12.9 (0.1)	[12.8, 13.0]
5.0	3	166.1 (6.8)	[158.4, 173.7]	15	3	-5.7 (0.6)	[-6.3, -5.0]
7.5	3	60.9 (3.0)	[57.6, 64.3]	20	3	-6.0 (0.6)	[-6.7, -5.2]

When a test strip is stored in 20 mg/mL BSA blocking solution, the maximum amount of BSA that can be retained within the test strip is 2,000  $\mu\text{g}$  assuming there is no physical adsorption to the strip (a wet paper strip contains a liquid volume of approximately 0.1 mL). Within 2.5 minutes of washing, the concentration of removable BSA within the test strip in the 5-mL vial was 246.3  $\mu\text{g}/\text{mL}$  or 1,231.7  $\mu\text{g}$ . The concentrations continued to decrease over time, reaching a concentration below the modified Bradford assays’s detectable limit in 15 minutes.





*Figure 1*  
Depletion of BSA Mass From Test Strip at Different Wash Durations (shown with standard error bars).

#### *Determination of Antigen Specific Binding*

The individual mean test strip mass after aspiration was 0.1538 g. Three active and three reference test strips were each exposed to a 413 µg antigen/mL solution (7.0 PBS) for two hours (0.0998 mL of antigen solution was applied to each test strip; the mean time-zero test strip mass was 0.2537 g). On average, 0.0190 mL of liquid volume per test strip was lost due to evaporation, giving a final mean test strip mass at the end of incubation of 0.2347 g. The total volume of liquid remaining in or on the test strip was 0.1902 mL.

After the two-hour test strip testing period, the mean antigen concentration of the solutions dropped to 242.2 (SD = 6.4) and 366.4 µg/mL (SD = 2.1) for the active and reference test strips, respectively. The concentration of unbound antigen on the reference test strip was greater than the active test strips (two-sample one-sided t test assuming unequal variances,  $t(2) = -18.51$ ,  $p = 0.0015$ ).

*Table 4*

Statistical Analysis of Antigen Concentration in Applied Test Solution After Two Hours During Test Strip Testing and Bound Antigen (Specifically and Nonspecifically Bound)

Test strip	Unbound Antigen Concentration (µg/mL)			Bound Antigen (µg)		
	n	Mean (SD)	95% CI	Mean - A	Mean - B	Mean - C
Active	3	294.2 (6.4)	[287.0, 301.5]	17.5 ± 0.3	-14.7 ± 0.3	8.1 ± 0.3
Reference	3	366.4 (2.1)	[364.0, 368.7]	11.6 ± 0.3	-28.4 ± 0.3	0.0 ± 0.3
Difference (Specifically Bound Antigen)				5.8 ± 0.4	13.7 ± 0.4	8.1 ± 0.4

Note. A = Method A; B = Method B; C = Method C.

Three possible methods of mixing were used to calculate the amount of antigen bound to a test strip. Methods A and B determine the lower and upper limits of possible antigen binding.

Method A: Assuming the applied antigen in the 0.0998-mL sample could not mix with the residual liquid in the test strip's pores, the mass of bound antigen to the active and reference test strips was 17.5 and 11.6  $\mu\text{g}$ , respectively. This results in 5.8  $\mu\text{g}$  of antigen being specifically bound to antibody in the active test strip.

Method B: Assuming complete and uniform mixing of the antigen in the applied sample with the residual liquid in the test strip's pores (a total volume of 0.1902 mL), the mass of bound antigen to the active and reference test strips was -14.7 and -28.4  $\mu\text{g}$ , respectively. This results in 13.7  $\mu\text{g}$  of antigen being specifically bound to antibody in the active test strip.

When applying Method B, the major assumption is that the residual liquid within the pores of the test strip is completely permeable to the antigen molecules. If the test strip pores are too small for the antigen molecule to penetrate, but readily available to water molecules, Method B would greatly overestimate the volume of liquid available to the antigen. As a result, the mass of remaining antigen in solution will be overestimated and the bound mass would be negative. These results were seen when Method B was applied to the antigen binding data. Method A could also overestimate the working volume, but not as much as Method B. Method B results are also unrealistic since the antigen is approximately 50 times larger than water molecules and a portion of the residual water volume within the paper strip is likely contained in pores too small for antigen penetration.

Method C: Instead of assuming a mixing extent as in Methods A and B, we assume zero antigen is bound to the reference test strip (corresponding to zero nonspecific binding), allowing the estimated maximum liquid volume containing the antigen to be calculated. This method results in the—likely too low—minimum amount of nonspecific antigen binding (0.0  $\mu\text{g}$ ) to the active and reference test strips. To be more precise, a pore size distribution of the paper would be needed to quantify the fraction of pore volume within the paper strip that is available for antigen penetration. However, even this would not be accurate as water swells the paper matrix and the porosity test would be conducted in the absence of water.

In addition to the lack of applicable information on the paper porosity, the additional physical modifications to the paper strip during manufacturing could restrict the movement of molecules through the paper strip. Setting the initial applied antigen mass equal to the final unbound antigen mass in the reference test strip provides a total liquid volume of 0.1126 mL available to the antigen. This volume would give an unbound antigen mass of 33.1  $\mu\text{g}$  and 41.3  $\mu\text{g}$  for the active and reference test strips, respectively. In this analysis, 8.1  $\mu\text{g}$  of antigen was calculated to be specifically bound to the active test strip while 0.0  $\mu\text{g}$  was nonspecifically bound to the active or reference test strips. The realistic amount of antigen specifically bound to the active test strip is likely between the values calculated by Methods A and C, 5.8 and 8.1  $\mu\text{g}$  of antigen per test strip.

### *Retention of Antibody-Binding Capacity*

The total mass of antigen binding to the test strip represents nonspecific and specific binding. The antigen binding difference between the active and reference test strips represents the specific binding of the antigen to the active test strip. This difference was between 5.8 and 8.1  $\mu\text{g}$  per test strip depending on the assumptions used (Method A and C).

The amount of antibody incorporated in the crosslinking manufacturing step in the active test strip was between 37.1 to 41.3  $\mu\text{g}$  depending on the assumptions used in the calculation (Method A and B). However, this could be a combination of crosslinked antibody and physically bound antibody. Later washing steps in the manufacturing process can remove any physically bound antibody, so the amount of antibody incorporated into the final test strip could be lower than when measured immediately after crosslinking. Assuming the antibody amounts presented above are either crosslinked or irreversibly, physically bound to the test strip with no reversible binding, the mass of antigen bound per antibody mass (using means) would be 0.177  $\mu\text{g}$  antigen/ $\mu\text{g}$  antibody. Sigma-Aldrich reports in the certificate of analysis that the active antibody binds 0.8 mg antigen per mg of antibody. Therefore, the active antibody retained around 22% of the manufacture's reported antigen-binding capacity.

### **Discussion**

The washing apparatus and procedure used to wash test strips was sufficient in completely removing loosely bound protein from the test strips. The minimum washing time was found to be 15 minutes when the peristaltic pumps were set to the highest flow rate.

The modified Bradford assay technique demonstrated, during the test strip crosslinking step, that 82% to 92% of the applied antibody mass was incorporated. The amount of binding for the reference antibody and the active antibody to the paper strips were nearly identical (< 3% difference), which is an important finding from this study as all test strips (active and reference) need to be comprised of the same amount of constituents. In this determination it was assumed that all the antibody that was depleted from the solution crosslinked to the test strip. Some of this antibody could have been physically bound and could have been removed later in manufacturing processing steps. However, what is ultimately important is that amount of active antibody contained and available in the active test strip and the amount of reference antibody contained and available in the reference test strip are the same after all downstream processing is complete. Knowing that they are essentially identical during the crosslinking step is important and this has been demonstrated in this study. Also, the two-hour incubation period was shown to be sufficient for most of the applied antibody (82 - 92%) to crosslink or physically bind to the test strip.

When the antigen solution was applied to the test strips, the active test strip bound more antigen than the reference test strip by 5.8 to 8.1  $\mu\text{g}$ , indicative of antigen specific binding. The active antibody's certificate of analysis from the manufacture indicates that this antibody binds a maximum of 0.8  $\mu\text{g}$  of antigen per  $\mu\text{g}$  of antibody. With 37.1 to 41.3  $\mu\text{g}$  of active antibody contained in each active test strip the current manufacturing process retains 22% of the active antibody's maximum antigen-binding capacity. ELISA methods generally use physical

(noncovalent) adsorption of monoclonal antibodies to polystyrene surfaces which typically retains much less than 10% of the maximum antigen-binding capacity.<sup>28,29</sup>

Since we do not know whether the antigen concentration (413 µg/mL) was high enough to saturate the incorporated antibody binding sites, the demonstrated 22% antigen-binding activity may represent a minimum and the actual could be higher. Additionally, if some of the antibody on the test strip was only physically bound and lost in later manufacturing steps, the antibody mass in the prepared test strip would be lower and the retained antibody antigen-binding activity would have been higher. The findings are significant and indicate that the design of the processing steps and conditions are achieving desired outcomes.

Using Method C for the antigen binding calculations showed that only 10% to 12% of the liquid volume within the paper strip pores is available for antigen penetration. This suggests that the antibody may be crosslinked primarily at the surface of the test strip, or that the crosslinking may severely restrict access of the antigen to liquid spaces within the test strip.

## Conclusions

This disposable point-of-use immunobiosensor is progressing through early stages of development. The findings of this work through the design and use of a modified Bradford assay technique have contributed to a better understanding of the test strip manufacturing process and structure of the test strips used in the study. The information gained regarding the efficiency of antibody crosslinking to the paper strip, percent activity retained by the antibody, and corresponding antigen binding at the antigen test solution concentration evaluated are all useful in adjusting future manufacturing conditions and in improving the test strip. The modified Bradford assay used in this study (and future studies) sheds light on how test strip manufacturing steps and design affect antibody incorporation within the test strip and help determine required test strip washing times. This information is also useful in establishing directions for future test strip designs.

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