Low-Cost Diagnostics: Using Paper as a Material and Pens as an Instrument

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Honors Thesis

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**ABSTRACT:** Lateral flow immunoassays (LFIs) are equipment-free tests that produce results quickly using small sample volumes. Colored lines appear as the test runs, indicating the presence of a biomarker. LFIs are ideal in a variety of settings. Development of these assays can be complicated for small team operations, and tests are not sufficiently adaptable for low resource settings. If robust point-of-care tests can be developed on site, they can expand the reach of global diagnostics, improving health around the world. We report a simple empowering LFI (seLFI) that only requires treated printer paper and a plastic backing card. This eliminates the use of nitrocellulose membrane, sample pad, conjugate pad, absorbance pad, and all the required layering during assembly. Additionally, test and control line antibodies can be applied to this treated paper by a rollerball pen, allowing on site preparation of the seLFI. The seLFI is a low-cost, labor-saving, single-sheet diagnostic that performs comparably to commercially available LFIs. These tests could assist small team operations in developing versatile tests to use on site, or to resolve complications during commercialization. They could also be used in low-resource or at-home settings to personalize tests before running them. We present the proof-of-concept seLFI to improve the accessibility, robustness and versatility of diagnostics research.
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INTRODUCTION

Across the globe, regardless of age, race, and condition of living, people struggle to overcome or live with health challenges. According to the World Health Organization, the number of patients with diabetes has risen to 422 million, with 3.7 million deaths caused by high glucose per year. The World Health Report indicates that 50,000 people die of infectious diseases daily, many of which could be prevented or cured for just one dollar a person. Approximately 76,000 women and 500,000 babies die worldwide from preeclampsia and hypertensive disorders yearly. In developing countries, women are seven times more likely to develop preeclampsia than women in developed countries. The World Allergy Organization estimated in 2011 that 30-40% of the world’s population experiences an allergy to one or more allergens. In a world of chronic disease, infectious agents, allergies, intolerances, and health complications, the need for diagnostic tests is becoming ever greater. The World Health Organization has developed a standard for diagnostic tests called the ASSURED criteria: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users. Diagnostic tests following these criteria allow healthcare providers with any range of resources to participate in improving the health of their patients.

In many situations, lateral flow immunoassays, or LFIs, are used to satisfy the ASSURED criteria, bringing diagnostic tests to even the most resource-restricted clinics. These equipment-free tests produce results in 10-20 minutes and use small sample volumes. LFIs are easy to run, and the results are straightforward to interpret; colored lines appear as the tests run, indicating the presence of a biomarker in a sample. Although traditional LFIs seem ideal for low-resource settings, additional challenges remain. By definition, low-resource areas struggle with poverty, and maintaining a strong medical front against epidemics is inherently expensive. While LFIs are manageable for first-world patients and clinics to purchase, affording these pre-assembled products can be difficult for small team operations globally.

In any setting, a diagnostic test needs to be non-invasive and fast, delivering quick results at the point of care (POC). In low-resource settings, a clinic might be without advanced training, equipment, or electricity, so it is critical that tests are easy to understand, run, and interpret. To reduce costs in low-resource settings, the tests also need to be easy, straightforward, and inexpensive to assemble; lowering the manufacturing costs reduces the cost of the test. To manufacture traditional LFI tests, a well-equipped lab would have a striper (a machine that immobilizes antibodies to nitrocellulose membrane), a laminator (a machine that aids the assembly of the layered lateral flow cards), and a test shear (a machine that cuts laminated cards into individual tests). A striper costs anywhere from $6,500 to $46,500 (ClaremontBio and BioDot), along with $6,100 to $8,625 for a laminator (BioDot and Kinematic Automation), and $13,600 to $16,790 for a test shear (BioDot and Kinematic Automation). On average, the start-up cost of a small-scale lateral flow research lab is $47,520 for this equipment and materials like nitrocellulose membrane, plastic backing cards, conjugate, sample and absorbance pads.

For small team operations, especially those new to diagnostics research, this up-front cost that is almost...
too great. For labs and clinics in low-resource settings, this equipment is too expensive, leaving them to source diagnostic tests from larger companies that were able to afford the start-up cost. Making assay development and diagnostic test manufacturing more accessible to these groups would promote good health practices, research, and scientific standing in these areas.

We introduce a new LFI platform that lowers the barrier of assay development and manufacturing for low-resource labs and clinics, as well as for small team operations. This platform, referred to as the “simple empowering lateral flow immunoassay” (seLFI), uses chemically treated paper attached on a plastic backing support, reducing the many layers of a traditional LFI to a single sheet of paper. Like the traditional LFI, the seLFI uses capillary action, conjugated antibodies, test and control lines, and a greatly simplified chase buffer to detect a biomarker.

We report the development of the seLFI, an LFI whose manufacture only requires treated printer paper and a supportive backing. This eliminates the need for nitrocellulose paper, sample pad, conjugate pad, absorbance pad and all assembly associated with these layers. Additionally, test and control line antibodies can be applied to this paper by an antibody pen, making the seLFI both adaptable and straightforward to develop. The seLFI is a low-cost, labor-saving, single sheet of treated paper that produces results comparable to commercially available LFIIs. In this paper, we describe a seLFI test developed to detect hCG in different sample matrices (spiked human urine, fetal bovine serum) at levels of 1:10^3 in under five minutes. These tests could assist individual researchers and small team operations in developing personalized, on-site diagnostics. We present the proof-of-concept seLFI to improve the production, robustness, and versatility of diagnostics in small team and low-resource operations.

A traditional LFI is made up of several layered materials adhered to a plastic backing card (Figure 1). These materials consist of a sample pad, a conjugate pad, a nitrocellulose membrane, an absorbance pad, and often require tape allowing compression between layers to facilitate flow (Fenton, et al.). Each of these layered materials are customizable,
with different types of materials to choose from, with varying treatments that can be applied. Often, simply changing the type of material or the treatment applied to it can determine if the LFI runs successfully, or if it fails. The materials can vary in size and in overlap, with some tests being long, others being thin, some with their layers overlapping 2.5 mm, 1 mm. Each test is different, with no simple, streamlined pathway of development. While developing an LFI, labs experiment with these many variations, and expend two valuable resources: time and funding. Manufacturing developed tests requires additional time and funding.

To streamline time and funding, we propose reducing the layers of the LFI from at least five layers to two: a plastic backing card and treated paper. Eliminating the sample pad, conjugate pad, nitrocellulose membrane, absorbance pad, and tape reduces the cost of materials significantly. The time spent working through each of these materials to find the best fit can be dedicated to developing the test in other areas. Manufacturing tests no longer involves overlapping layers by exact measurements and pressing the overlaps together with tape or a cartridge, but rather securing treated paper to a plastic backing card. The expensive machines used to stripe the tests are replaced by a rollerball pen filled with antibody ink. Treatment of each material goes from a multitude of options to one: modifying the paper to behave like nitrocellulose membrane.

■ METHODS

Progression to “Sandwich Model”

Traditional LFI tests follow the “sandwich model”, where a conjugated antibody binds to the target biomarker. This conjugated antibody-biomarker complex binds to unconjugated antibody that is secured to the nitrocellulose layer as the test line. The test line constitutes unconjugated antibodies that recognize the conjugated antibody. The three proteins (unconjugated antibody, antigen, and conjugated antibody) form what is called the “sandwich model” and is the clinically relevant goal when developing a diagnostic test.

To begin seLFI development, we focused on the simplest version of this clinically relevant model, termed “tier one”. A tier one test was purposed to answer two questions:

1. Would protein remain bound to the modified paper test?
2. Could the bound protein withstand the friction of fluid flow, or would capillary action dislodge the bound protein?

To answer these questions, we built a minimalistic test with only one protein. This was done by modifying printer paper, either by nitration or by aldehyde-functionalization, securing it to a plastic backing card, and dividing it into test strips. The test strips were striped with conjugated antibody (α-hCG beta antibody in goat, gold colloid, Fitzgerald) and were subjected to capillary flow of 5% dry milk TBS-T buffer. Following the tier one test, a tier two test was developed.

A tier two test built upon this model, adding a protein to the test. Tier two would answer the following question:

1. Could a test or control line capture antibodies or other proteins as they flow past in a buffer medium?
2. If so, will the captured proteins remain bound as additional sample and buffer clears the test or control lines?

Tier two tests were made with both nitrated printer paper and aldehyde-
functionalized printer paper, then compared to see which paper treatment performed best. Modified printer paper was striped with hCG protein (EMD Millipore) and allowed to dry. 5% dry milk TBS-T buffer spiked with conjugated antibody (α-hCG beta antibody in goat, gold colloid, Fitzgerald) was used to induce capillary action. Since we stopped using nitrated printer paper after tier two, the details of this method are included in the section dedicated to nitrated printer paper.

Tier three was built as a final phase preliminary test, answering the standard questions:
1. Could a line of antibodies capture a protein-conjugate complex?
2. Could a line of antibodies capture the conjugated antibody?

At this point in our research, nitrated printer paper had ceased to be a focus, whereas aldehyde-functionalized printer paper became our first choice for the seLFI. Therefore, our tier three tests were developed on aldehyde-functionalized printer paper only, and not nitrated printer paper. These tests were first made with only a test line: a stripe of α-hCG (α-hCG alpha antibody in goat, MyBioSource) that would be met with a complex of hCG (EMD Millipore) and conjugated antibody (α-hCG beta antibody in goat, gold colloid, Fitzgerald). We then developed a tier three test with only a control line: a stripe of anti-host antibody (rabbit anti-goat, Fitzgerald, MyBioSource) that would be met with conjugated antibody (α-hCG beta antibody in goat, gold colloid, Fitzgerald). Since tier three was our clinically relevant goal, the details are outlined in the following section dedicated to aldehyde-functionalized printer paper.

### Nitrated Printer Paper

Nitrocellulose membranes use hydrophobic interactions to bind to proteins. These hydrophobic interactions are strong, and once bound, only extreme measures can remove proteins from nitrocellulose membrane. Therefore, when nitrocellulose membrane is striped with test and control antibodies, they remain fixed. The surrounding active sites on the membrane are blocked, and additional proteins, such as those in a biological sample, can flow through the membrane, only binding to specific antibodies with which they pair. Compared to nitrocellulose membrane, paper is a low-cost solution, but it must be modified to secure proteins and keep them from drifting as biological sample and buffer flow through the paper. Nitration was the first method explored to modify paper to act like nitrocellulose membrane. Equal parts of nitric and sulfuric acid were carefully combined and stored at low temperatures in a small glass vial. Printer paper (75 g/m², 92 bright, Target) was attached to a plastic backing card and cut into 5.0 mm x 34.5 mm strips. The chilled acid mixture was pipetted across the middle zone of the paper strips to create a fine line (2.0-3.0 mm) that would hold the test protein. After the acid mixture was deposited and allowed to absorb, the paper strips were run under continuous water for five minutes, then incubated with sodium bicarbonate and water under shaking for an additional five minutes. After neutralization, the nitrated paper strips could dry at room temperature and were stored in a sealed bag with desiccant packs at room temperature.

In the first phase of research, hCG was acting as the test protein, with conjugated α-hCG acting as the biomarker spiking the buffer. This was a “first-step” toward a more clinically
relevant “sandwich” model, with antibodies as the test and control proteins, and unconjugated proteins acting as the biomarker. Initially, the tests were bare-bones simple, involving only hCG and conjugated α-hCG. To prepare the test strips, 1.0 μL of hCG at 1.0 mg/mL (the test protein in this model) was pipetted along the nitrated zone and allowed to dry and bind. In order to prevent false positives, the active sites of the nitrated zone were blocked by pipetting 3 μL of a 5% solution of dry milk in TBS-T onto and around the edges of the test zone. After drying, the tests were ready to run.

Aldehyde-functionalized Printer Paper

Another successful method of securing proteins to paper was developed by Badu-Tawaiah et al. This method takes cellulose and creates aldehyde groups through a process of incubating the paper in a solution of KIO₄. Those aldehyde groups can then form Schiff bases with proteins that contact the paper, similar to nitrocellulose. In this method, one sheet of printer paper was placed in a 0.03 M solution of KIO₄. The paper was then soaked for 2 hours at 65°C. After treatment, the paper was rinsed three times in fresh deionized water, and then allowed to dry overnight at 35°C.

Once the paper was dry, antibodies were added to the paper in test and control lines. The test line antibodies and control line antibodies were placed directly onto the aldehyde functionalized paper using a straight edge to guide the line and a 10 μL syringe to apply the antibody (1.0 mg/mL). Later, syringes would be replaced with antibody pens, which are discussed hereafter. Since the entire paper’s surface was functionalized after treatment, the strips were blocked with 5% dry milk blocking buffer in water to ensure that the paper would not have open active sites. After the blocking, the paper was dried, then placed onto a plastic backing card. Once attached to the card, the paper was cut into 5.0 mm x 34.5 mm strips. The process of creating aldehyde functionalized paper is simple and has the potential to be delivered to the end user ready to be striped and blocked.

To run the test strips, 90.0 μL of 5% dry milk solution in TBS-T was added to 14 2.0 mL microcentrifuge tubes. Seven of the tubes acted as positive controls: six for the seLFI, and one for the commercially available pregnancy test. To these seven tubes, 1.0 μL of hCG (1.0 mg/mL) was added to each tube separately and mixed gently with a pipette. The other seven tubes acted as negative controls, with six for the seLFI, and the seventh for the commercially available pregnancy test. 10.0 μL of gold nanoparticle (AuNP) conjugated goat α-hCG antibodies were added to each tube separately and mixed gently with a pipette. After preparing these samples, test strips were placed into each tube and allowed to run via capillary action.
Antibody Pens

Aldehyde-functionalized paper proved to be a strong, flexible, inexpensive alternative to nitrocellulose. Striping this paper with test and control antibodies would traditionally be done with an automated dispenser. However, the goal of this project excluded the use of expensive equipment and electricity, since neither are currently feasible in low-resource areas. Even other low-cost options such as using a printer or a simple X-Y plotter required electricity, and it became clear that the target demographic would require a more manual technique. The best way to precisely deliver a substance to a surface without electricity is with a writing instrument. Research led to filling felt-tipped markers, brush pens, ballpoint pens, and paint pens with an antibody solution “ink”, with the most success of delivery being found in a rollerball pen. These rollerball pens can be filled with virtually any antibody ink and are able to draw lines of antibodies onto the aldehyde functionalized paper, producing the control and test lines for many different kinds of tests. We found that J. Herbin refillable rollerball pens (Figure 2) were best suited for delivering antibody ink since they used a wick to draw up the ink and used a refillable piston cartridge to hold ink (Kawe co Mini Piston Converter). This made it easy to test, clean, and reuse the pen with different types of antibodies. This method of delivery promotes user-specific and diverse diagnostic tests for a variety of settings—including low-resource settings and small team operations.

To test feasibility, the piston cartridges were filled with rabbit α-goat antibodies and goat α-hCG antibodies. The control pen (goat α-hCG) was used to write on aldehyde functionalized paper and nitrocellulose. Once the protein ink had dried, the blots were blocked together with 5% dry milk TBS-T buffer at 25°C while shaking for 1 hour. After rinsing, the blots were incubated in similar conditions with TBS and 60 μL hCG for 1 hour. After this incubation, the blots were

Figure 2. Antibody Pen
Pictured is a sequential explosion of the antibody rollerball pen. A) shows the pen completely assembled and capped. B) shows the pen uncapped, exposing the tip of the rollerball pen. C) shows the body unscrewed from the tip housing, showing how the piston cartridge attaches securely to the tip housing, connecting to the wick. D) shows the depressed piston cartridge detached from the tip housing, exposing the faint outline of the white wick in the tip housing. E) shows the same, but with the piston cartridge completely extended. The piston cartridge holds the antibody ink, which is carried to the tip of the rollerball pen through a wick when the two are properly connected. Each part can be cleaned and reused with new or different antibody when the user desires.
rinsed and incubated with 400 µL AuNP-conjugated goat α-hCG antibodies under similar conditions for 1 hour. The blots were then rinsed and dried.

Additional tests were done to determine the antibody pen’s longevity. The antibody pen piston cartridge was filled with 100 µL of rabbit α-goat antibodies, secured to the pen, and then was used to draw several straight lines of antibody ink on a single sheet of paper until the pen ran dry. The paper was then blocked for 1 hour in 5% dry milk solution in TBS-T, washed in deionized water, then incubated with 400 µL AuNP-conjugated goat α-hCG antibodies in TBS under shaking for 1 hour. The paper was removed, rinsed with deionized water, and allowed to dry. The observed lines were measured for length, determining the approximate number of tests 100 µL of antibody in the antibody pen could make.

Testing in Urine

Although the seLFI had proven its potential in a protein-spiked sample buffer, a clinically relevant diagnostic would need to perform in biological samples. Testing the seLFI in biological samples began with urine, due to simplicity of training, collection, and storage. To run the test strips, 90.0 µL of urine were added to eight 2.0 mL microcentrifuge tubes. 1.0 µL of hCG (1.0 mg/mL) was added to the first four tubes separately and mixed gently with a pipette, constituting our positive samples. An additional positive control was performed with a commercially available pregnancy test, run with the sample in the fourth tube according to the package directions. The remaining four tubes were left without hCG, constituting the negative samples. An additional negative control was run using a second commercially available pregnancy test. To the remaining six tubes (both positive and negative samples), 10.0 µL of AuNP-conjugated goat α-hCG antibodies were added to each tube separately and mixed gently with a pipette. After preparing these urine samples, previously prepared aldehyde functionalized test strips were placed into each tube and allowed to run via capillary action.

Testing in Serum

Another clinically relevant goal was to test the seLFI in serum. 90.0 µL of fetal bovine serum (FBS) were added to eight 2.0 mL microcentrifuge tubes. 1.0 µL of hCG (1 mg/mL) was added to the first four tubes separately and mixed gently with a pipette, constituting the positive samples. An additional positive control was performed with a commercially available pregnancy test, run with the sample in the fourth tube according to the package directions. The remaining four tubes were left without hCG, constituting the negative samples. An additional negative control was run using a second commercially available pregnancy test. To the remaining six tubes (both positive and negative samples), 10.0 µL of AuNP-conjugated goat α-hCG antibodies were added to each tube separately and mixed gently with a pipette. After preparing these FBS samples, previously prepared aldehyde functionalized test strips were placed into each tube and allowed to run via capillary action.

Limit of Detection

A lateral flow immunoassay must detect a biomarker; furthermore, it must detect the correct levels of that biomarker in the sample. Up until this point, we had been loading a large amount of biomarker
(1 µL of 1 mg/mL hCG) into sample buffer to build a working preliminary seLFI. To establish a working credible seLFI, we had to determine the limit of detection. The goal was to observe the lowest level of biomarker the seLFI could detect, especially in a way that the lowest level could be read by the human eye. Our desired outcome was for the seLFI to detect the concentration of hCG in urine during pregnancy, or 2.5 µg/mL.

To prepare the test runs, a serial dilution was set up in six dilutions. 90 µL of 5% dry milk solution in TBS-T was added to seven 2 mL microcentrifuge tubes. 10 µL of hCG (1 mg/mL concentration, or 1:1) was added to and mixed in the first dilution, resulting in 1:10 hCG concentration. 10 µL of solution was removed from the first dilution, added to the second microcentrifuge tube and gently mixed, resulting in a 1:10^2 hCG concentration. This process was repeated until six tubes contained hCG ranging from 1:10 to 1:10^6 concentrations. The final tube was left without hCG and served as a negative control.

This serial dilution was repeated three times in additional microcentrifuge tubes: two of the sets of serial dilutions acted as replicates for the original dilution, and one set of serial dilutions would be used to run commercially available pregnancy tests as a positive control. These commercially available tests were run according to the package directions, using each tube separately for a total of six positive control tests. The final tube, having no hCG, served as the negative control.

In the remaining three sets of tubes, 10 µL of AuNP-conjugated goat α-hCG was added to and gently mixed in each separate dilution. After preparing these dilution samples, previously prepared aldehyde functionalized test strips were placed into each tube and allowed to run via capillary action.

This experimental set-up was repeated to test the seLFI’s limit of detection in urine, replacing the milk chase buffer volume with the same volume of urine, keeping all other conditions the same.

■ RESULTS

Nitrated Printer Paper

In hopes of simplifying and reducing the cost of the traditional LFI by eliminating the sample pad, conjugate pad and absorbent pad, a low-cost format that was friendly to low-resource areas, the single-sheet seLFI, was developed. This experiment tested whether the seLFI format could support stationary antibody-antigen binding at the “test line”, like the traditional LFI. After nitrating the printer paper, striping with antibody, and performing positive control seLFI tests, some encouraging results followed. The running buffer was entirely absorbed by the seLFI test strip, and capillary action took the conjugated α-hCG past the test line of hCG protein, which was bound to the modified area of the test strip. Binding between antigen and conjugated antibody occurred, similar to a traditional LFI sandwich format. Figure 3 shows the genuine promise of performing a diagnostic test using only one layer of treated printer paper. The nitrated zone acted as a pseudo test line (the first antigen and conjugated antibody model
used was not a true test line, presenting a visual positive result. These visual results indicated that the modified areas of the paper functioned akin to nitrocellulose; stationary test lines on printer paper can be maintained even after the introduction of running buffer and sample flow.

Despite the preliminary success of these elementary test results, several points were concerning. Visually, the results were diffuse and nonuniform, making it difficult for technicians to distinguish a positive result from a negative result. Each test produced a dramatically different result, presenting a challenge to the tests’ reliability. On several of the tests, the nitrated zone behaved as a hydrophobic barrier, encouraging the sample to flow around the zone, rather than directly through it. Notwithstanding the practice of blocking around the nitrated area after striping with antibody, negative tests were inconsistent (not pictured). Outside of test performance, test manufacture was risky and inconvenient, due to the use of strong acids. With these challenges and dangers, it was decided to pursue an alternative to nitrated printer paper.

**Aldehyde-functionalized Printer Paper**

In a safe and convenient alternative to nitrated printer paper, the method of Badu-Tawaiah et al. was used to aldehyde-functionalize printer paper. Printer paper was treated with KIO₄, separate lines of test and control antibodies were striped, and the paper was blocked and cut into test strips. Running the tests with positive and negative samples (5% dry milk solution in TBS-T with and without hCG) produced clear, uniform, and legible results (Figure 4). These results demonstrate the seLFI’s ability to compare to traditional LFI results, in speed, reliability, and specificity.

In the positive seLFI results (Figure 4: A), the milk chase buffer, AuNP α-hCG, and hCG were drawn up via capillary action, crossing the entire test, including the test and control lines. The hCG/AuNP α-hCG complex remained bound to the test line, and the AuNP α-hCG remained bound to the control line, resulting in the visual result of two pink lines. In the negative seLFI results (Figure 4: B), the chase buffer and AuNP α-hCG ran through the entire test, crossing both the test and control lines. There was no hCG/AuNP α-hCG complex, so nothing bound to the test line, but the AuNP α-hCG remained bound to the control line, indicating a valid test.
This yielded the visual result of one pink line. The commercially available pregnancy tests (Figure 4: C) show the negative control (left) and positive control (right). These tests show an intensity of color in the results and a low background that surpassed the seLFI tests. Though the seLFI tests exhibit potential in accuracy, specificity, and visual readability, optimization was needed.

Antibody Pen

The antibody pen was used to draw letters on to aldehyde-functionalized paper using goat α-hCG antibody ink. These markings could be seen as wet lines until they dried; after drying, the writing disappeared entirely. The consequent steps in the experiment—blocking, incubating with hCG, then incubating with AuNP α-hCG—were intended to facilitate a “sandwich” complex between the invisible goat α-hCG that was drawn onto the paper, the suspended hCG, and the suspended AuNP α-hCG. This test would determine if the antibody pen allowed for proteins to pass through the system without being damaged, and if the delivery would be detailed, even, and precise (Figure 5).

Figure 4. Aldehyde-functionalized seLFI Strips.
Half-strips were prepared with a 0.03 M solution of KIO₄ and heat, then striped with lines of control (top line) and test (bottom line) antibodies. The striped paper was blocked and cut into strips, then the individual tests were run. A) Positive tests were performed with 30 μL of 5% dry milk buffer, 10 μL of gold nanoparticle conjugated α-hCG, and 1 μL of 1 mg/mL hCG. Results are clear, with both the control and test lines showing specific binding. B) In turn, the negative tests were performed under similar conditions, with the exception of hCG. As expected, only the control line showed specific binding, with a blank test line indicating the absence of hCG. C) Additional commercially produced positive controls were run under similar conditions to verify the positive and negative seLFIs.

Figure 5. Antibody Pen Precision and Efficacy
To determine if the antibody pens would write evenly and precisely without damaging the antibodies in the ink, the piston cartridge was filled with rabbit α-goat antibodies. It was then used to write on aldehyde functionalized paper and nitrocellulose. Once the protein ink had dried, the blots were blocked together with 5% dry milk TBS-T buffer at 25°C while shaking for 1 hour. After rinsing, the blots were incubated in similar conditions with TBS and 60 μL hCG for 1 hour. After this incubation, the blots were rinsed and incubated with 400 μL AuNP-conjugated goat α-hCG antibodies under similar conditions for 1 hour. The blots were then rinsed and dried. A and B show results on nitrocellulose, while C and D show results on aldehyde-functionalized paper. A and C were written by technician A, and B and D were written by technician B.
These completed tests presented clear, uniform, legible lines. For both aldehyde-functionalized paper and nitrocellulose paper, the antibody delivery is so detailed that the differences in handwriting between the two technicians can be identified. When run under identical conditions, the aldehyde functionalized paper produced a clearer, darker result than the nitrocellulose paper. The pen has been licensed to DCN Diagnostics and is currently being developed to be included in their LFI starter kits.

The second test determined the lifetime of 100 μL of antibody ink in the pen (Figure 6). After drawing, blocking, incubating, and drying, the lines were measured to project the total number of tests 100 μL of antibody could produce. A total of 5,588 mm was measured before the integrity of the line began to break up, leaving the rest unmeasured for the sake of quality control. This length would result in approximately 1,117 tests. These tests indicate that the antibody pen is feasible with the low-cost, machine-free manufacturing system required by a low-resource lab or clinic.

**Testing in Urine**

Testing the seLFI in urine would expand the platform from a research relevant diagnostic to a clinically relevant diagnostic. After running the hCG seLFI tests in milk buffer successfully, we reproduced the testing procedures for testing in urine, simply replacing the milk chase buffer with the same amount of urine (Figure 7). Production of the tests and the amounts of hCG and AuNP α-hCG remained the same as the tests run in milk chase buffer. The tests performed

![Figure 6. Antibody Pen Extinction Test](image)

**Figure 6. Antibody Pen Extinction Test**

This test was designed to better understand the lifetime of antibody ink in the pen. 100 μL of rabbit α-goat antibodies were used to fill the piston cartridge, then drawn out in straight lines until the antibody ink ran out. The paper was then blocked for 1 hour in 5% dry milk solution in TBS-T, washed in deionized water, then incubated with 400 μL AuNP-conjugated goat α-hCG antibodies in TBS under shaking for 1 hour. The paper was removed, rinsed with deionized water, and allowed to dry. The lines were later measured and broken down into an approximate yield of tests.

![Figure 7. Aldehyde-functionalized seLFI Strips in Urine](image)

**Figure 7. Aldehyde-functionalized seLFI Strips in Urine**

Half-strips were prepared according to the aldehyde-functionalization method. Positive tests (left) were performed with 90 μL of urine, 10 μL of AuNP α-hCG, and 1 μL of 1 mg/mL hCG. Results are clear, with both the control and test lines showing specific binding. Negative tests (right) were performed under similar conditions, without the addition of hCG. As expected, only the control line showed specific binding, with a blank test line indicating the absence of hCG. Commercially available (center) positive and negative controls were run under according to package directions to validate the seLFI results.
exceptionally well, revealing clear test and control lines in the positive samples, and clear control lines in the negative samples. The tests ran significantly faster in urine than they did in milk chase buffer: a quick 2 minutes compared to 20 minutes.

Testing in Serum
With encouraging results from testing in urine samples, the next step was to test the seLFI in fetal bovine serum (Figure 8). Like the urine tests, we simply replaced the milk chase buffer with the same amount of FBS, keeping all other variables consistent. These tests still showed promise, presenting consistent positive and negative results, and taking only about 2 minutes to complete.

Figure 8. Aldehyde-functionalized seLFI Strips in Fetal Bovine Serum
Half-strips were prepared according to the aldehyde-functionalization method. Positive tests (left) were performed with 90 μL of fetal bovine serum, 10 μL of AuNP α-hCG, and 1 μL of 1 mg/mL hCG. Results are clear, with both the control and test lines showing specific binding. Negative tests (right) were performed under similar conditions, without the addition of hCG. As expected, only the control line showed specific binding, with a blank test line indicating the absence of hCG. Commercially available (center) positive and negative controls were run under according to package directions to validate the seLFI results.
Limit of Detection

Finding the seLFI’s limit of detection would allow for a side-by-side comparison of the seLFI’s sensitivity and the sensitivity of commercially available tests. Ideally, the seLFI would be able to detect hCG at the level of $1:10^4$ in order to detect 2.5 ng/mL of hCG in a positive urine sample. The seLFI consistently bound hCG at concentrations of $1:10^2$ to $1:10^3$, with unreliable, faint binding at $1:10^4$ (Figure 9). Interestingly, a

![Figure 9. Aldehyde-functionalized seLFI Limit of Detection](image)

Half-strips were prepared according to the aldehyde-functionalization method. All samples were prepared in similar fashion, with 90 μL of sample in each tube and 10 μL of hCG used to make a serial dilution of values from 1:10 to 1:10⁶. These were repeated three times to make a total of four runs: three dedicated to testing the seLFI, and one dedicated as a control with commercially available pregnancy tests. A-C show results with milk buffer, with D as the control. E-G show results with urine, with H as the control.
concentration of hCG at 1:10 resulted in weak test lines as well. Acknowledging the inconsistent binding at 1:10 and 1:10⁴ and beyond, we determine the seLFI limit of detection to be between 1:10² and 1:10³. These results were consistent across all three trials for both milk chase buffer (Figure 9 A-D) and urine samples (Figure 9 E-H).

**DISCUSSION**

The aldehyde-functionalized paper is a low-cost, safe, flexible replacement for nitrocellulose. For small labs or clinics with low funding or few resources, purchasing manufacturing machinery to make tests for labs, or purchasing pre-made tests for clinics is financially challenging. Having an electricity-free instrument that could be used to make diagnostic tests for research or for clinical use would serve those populations well. The antibody pen delivers similar results at a small scale. The low price and simple maintenance and use of the pen lends itself well to diagnostic development, as well as fast test manufacture for in-field use.

Diagnostic tests use biological samples to detect the presence of biomarkers in the sample, becoming a tool to aid in diagnosis and treatment of diseases and conditions. Biological samples range from whole blood to serum, tears to saliva, urine to feces, and many more. The ability to detect biomarkers in biological samples was key to the success of the seLFI, allowing it to function as a diagnostic tool. Due to the presence of hCG in urine during pregnancy, it was encouraging to observe the seLFI’s results when tested in urine. A short running time with clear, legible results opens the possibility of developing a test for a different biomarker in a different sample type. Although urine has a connection to the biomarker used in this study, we chose to test for hCG added into serum during sample preparation. The purpose of these tests was to explore the possibility of testing in different types of samples, while still working with a biomarker and antibodies that we were familiar with. The results of the seLFI serum tests were likewise encouraging and led us to plan to test the seLFI in whole blood and saliva. The goal is to prove the seLFI platform in as many biological samples as possible, in order to establish its potential as a new, innovative, and versatile diagnostic platform.

In order to be an effective test, the seLFI’s sensitivity would need to achieve a limit of detection of 1:10⁴. In order to improve sensitivity, a few avenues will be pursued. The first would be to simply increase the amount of AuNP α-hCG used while running the test. This would saturate the available hCG, increasing the intensity of the test line, despite the lower levels of protein in the sample. Another avenue involves antibody-antigen pairings. With a fade-out at 1:10⁴ concentration of hCG, the issue is between the α-hCG, hCG, and AuNP α-hCG sandwich complex. Finding a variety of these proteins and would allow us to set up a protein matrix. This would involve a series of blot tests, each with the same α-hCG variety blotted onto a small square of aldehyde-functionalized paper, then blocked separately. Each of the blots would then be incubated with the same variety of hCG, then washed, then each blot would be incubated with a different variety of AuNP α-hCG. These blot matrices would be repeated, adjusting the varieties of α-hCG, hCG, and AuNP α-hCG appropriately until an ideal match is achieved.
discovered. This ideal match would indicate the proteins that have the highest binding affinity, increasing the amount of hCG binding to both $\alpha$-hCG and AuNP $\alpha$-hCG, strengthening the intensity of the test line. An alternative avenue would be to increase the concentration of $\alpha$-hCG on the test line, so as to capture more hCG-AuNP $\alpha$-hCG complexes, raising the level of sensitivity.

The possibility of a false-negative would need to be addressed, considering the weak, unreliable binding at concentrations of hCG at 1:10. To prevent a false-negative, protein interactions at concentrations of 1:10 would need to be studied and understood more thoroughly in order to plan experiments and adjust the test and its procedures.
REFERENCES


Please see supplementary information in A. Badu-Tawiah’s publication: http://www.rsc.org/suppdata/lc/c4/c4lc01239a/c4lc01239a1.pdf