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Letter

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Detection of Biomarkers of Periodontal Disease in Human Saliva Using Stabilized, Vertical Flow Immunoassays

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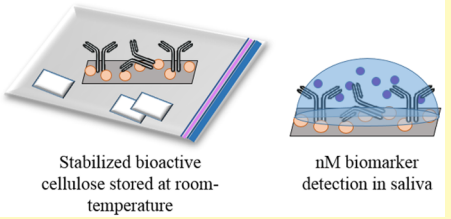
Supporting Information

ABSTRACT: We report methods for stabilizing cellulose-based immunoassays and using this platform to analyze human saliva. Stabilization treatments of immunoassays for matrix metalloproteinases (MMP)-8 and -9, biomarkers of periodontal disease, were conducted and compared, revealing that anti-MMP-8 and -9 capture antibodies could be stabilized with the addition of a 5% trehalose solution to the test zones, followed by drying in a vacuum oven. After stabilization, the paper devices retained equivalent binding activity to that of freshly prepared tests for 14 days—a time frame that enables US-based clinical testing of this diagnostic assay. A saliva pretreatment method was developed to remove viscous elements without reducing the concentration or binding activity of dissolved proteins. Immunoassays were stored in ziplock bags containing desiccant, and used to detect nanomolar concentrations of MMP-9 in human saliva across the relevant clinical concentration range. These methods and findings facilitate rapid, affordable validation studies of this and other biomarkers that are found in saliva using vertical flow immunoassays.



KEYWORDS: *diagnostics, low-cost, saliva, protein stability, periodontal disease, paper, polymerization*

Vertical flow, paper-based immunoassays for the detection of disease-indicating proteins have received much interest in recent years as potential tools for expanding access to reliable diagnostics and early disease treatment due to their ease of manufacture, low cost,[1−4] and self-driving flow.[4,5] We previously reported cellulose-based, vertical flow immunoassays for antigens in blood indicative of malarial infections, highlighting a potential for ultra-low-cost diagnostic assays that use polymerization reactions to provide rapid, high-contrast colorimetric readouts.[6,7] However, for practical applicability and generality, certain aspects of the approach remain as areas for advancement. First, the bioactive cellulose sensing layers in the immunoassay had to be prepared fresh and could not be stored before use. Second, the cellulose-based assays were useful in detecting biomarkers in serum, but similar capabilities in other human bodily fluids were not established. To be practical for clinical testing, cellulose-based immunoassays should be able to be pre-prepared and stored at ambient temperatures until needed. To be versatile for widespread clinical use, an ability to detect biomarkers in multiple human bodily fluids is also desirable.

While many paper-based assays are compatible with human serum or blood,[1,8,9] compatibility with saliva is an underdeveloped area of interest. Blood is a highly useful target medium for diagnostic assays, but the specifics of disease often make one bodily fluid preferable to another—for oral diseases, especially, saliva compatibility is desirable. Saliva contains important biomarkers of many diseases;[10−12] however, major challenges associated with its analysis include high viscosity and proteinaceous molecular assemblies that slow capillary flow through the device, variable flow rates, hindered transport of assay reagents, and aggregation of antigen detector molecules.[13] A goal of the present study was to develop methods to mitigate the effects of salivary mucins and other viscous, proteinaceous species before analysis with a cellulose-based immunoassay in a way that does not negatively affect the protein biomarkers in the saliva.

Periodontal disease was chosen as a model disease for this study. Periodontal disease arises from bacterial infections in the mouth, known to induce protein biomarkers that have been identified in human saliva.[14−16] It is a major cause of tooth loss in adults, due to soft tissue inflammation and bone loss, and has been linked to cardiovascular disease, osteoporosis, and diabetes.[15,16] Standard diagnostic practices of alveolar bone height and clinical attachment levels, while helpful, do not provide for patient-to-patient variation or identify risk for disease progression.[14,15] Clinical assessment of periodontal disease-indicating biomarkers has largely been focused on gingival crevicular fluid, which contains higher concentrations of disease biomarkers on average than whole saliva,[17] but is impractically difficult to collect in many clinical settings.[15] These issues could be addressed with whole saliva-based quantifiable biomarker tests to monitor at-risk patients during their appointments.

Matrix metalloproteinase-8 (MMP-8) and -9 (MMP-9) have been shown to be indicators of periodontal infections.[14−16]

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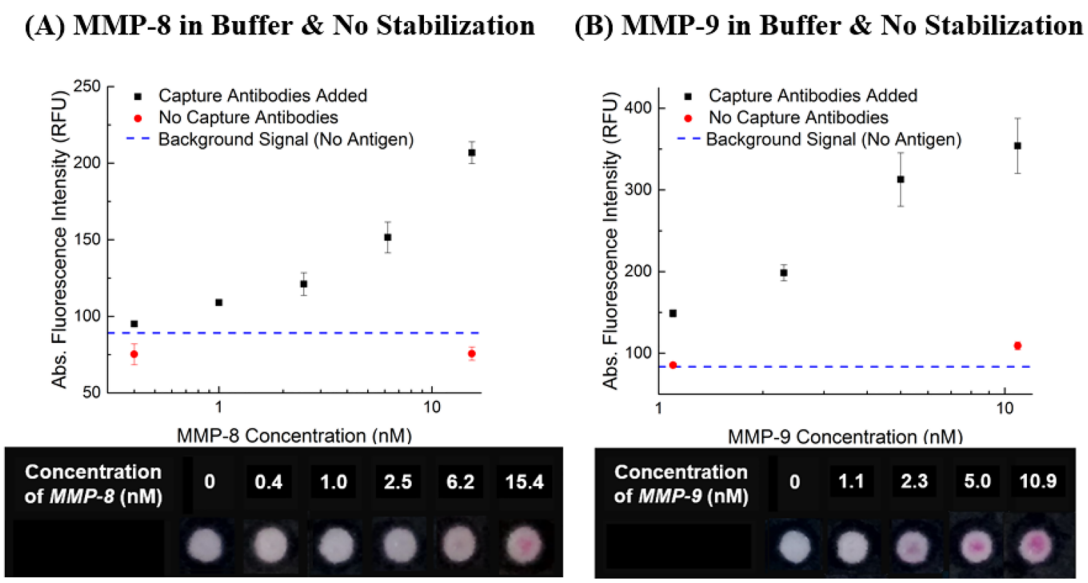

Figure 1. Fluorescence and colorimetric readout across the clinically relevant range for MMP-8 and MMP-9 assays with detection in phosphate buffered saline (PBS) buffer and no stabilization.

MMP-8 in the range of 0.4−15.4 nM (25−1000 ng/mL) and MMP-9 in the range of 1.1−10.9 nM (100−1000 ng/mL) in saliva constitute elevated levels that indicate disease.[15] In the present study, paper-based immunoassays for these two enzymes were developed and tested.

A sandwich immunoassay—where immobilized antibodies capture target biomarkers, and the capture event is detected by the binding of a second, reporter antibody to the captured biomarker—was developed that utilized eosin-based polymerization signal amplification for colorimetric visualization.[6] Protocols for the sandwich immunoassay and colorimetric detection via polymerization were performed as previously described for other biomarkers[6,7] and can be found in more detail in the SI. Stability was achieved through the addition of trehalose and drying antibody reagents onto paper assay wells for storage. Many studies have shown that the addition of sugars—especially trehalose—to both lyophilized and solubilized immunoglobins allows for months-long stability at elevated temperatures[18−20] due to a combination of sugars' hydrogen bonding and glass matrix formation abilities that stabilize the antibody in the absence of water.[21,22] In this study, trehalose was investigated for its stabilizing effects on antibodies immobilized in cellulose.

To test the immunoassay's ability to detect the two chosen biomarkers, MMP-8, MMP-9, and their respective capture and biotinylated reporter antibodies were obtained, and a paper-based sandwich immunoassay was performed with antigen dissolved in buffer (Figure 1A,B). Fluorescence microscopy as well as colorimetric signal amplification were used to quantify eosin-conjugated reporter molecules bound on each test zone.

It was found that this methodology could successfully detect MMP-9 across the clinically relevant range of elevated concentrations. MMP-9 assays maintained a larger fluorescence signal difference between antigen-containing samples vs negative samples than MMP-8 assays. A positive colorimetric signal was not visible for all clinically meaningful elevated concentrations of MMP-8. While severe infections would likely be detected, low level gingivitis may be missed if MMP-8 alone was tested. Because the signal amplification reagents were held constant in the two assays, we attribute the differences in detection limits to either the binding constants of the two antibody pairs, or the degree of biotinylation of the two different reporter antibodies, or a combination of the two.[23]

After assessing the diagnostic potential of the two MMP biomarkers, methods were investigated for stabilizing MMP-9 antibodies immobilized on paper. A 5% w/v solution of trehalose in 1× PBS was applied as a stabilizing agent to wells containing capture antibodies covalently bound to aldehyde-functionalized paper. The sugar−antibody solution was then dried onto the paper using various drying techniques (lyophilization, vacuum oven at 45 °C, air drying at 4 °C, and drying in a desiccator). Lyophilization and vacuum oven drying led to the best retention of antibody activity after storage in a desiccator for 2 weeks. The detection limit of immunoassays subjected to these top two treatments was tested, and it was found that vacuum oven drying treatment produced a higher contrast colorimetric readout (SI Figure S3), on average, than lyophilized test strips. Vertical flow paper assays that were stabilized with trehalose, vacuum-oven-dried, and stored in a desiccator for 2 weeks were compared to vertical flow paper assays prepared continuously and not allowed to dry by examining their ability to detect MMP-9 in buffer. The fluorescence signal due to eosin bound to the surface—an indicator of antigen binding and subsequent colorimetric detection via polymerization—showed that there was no significant change in signal when compared to MMP-9 immunoassays that were used continuously and not allowed to dry (SI Table S2). Vacuum oven-stabilized assays were also tested by storing them in a ziplock bag with desiccant pellets. When used to test samples of MMP-9 at a concentration within the dynamic range (4.3 nM), the assay readout was not significantly different after 14 days from the readout after 0 days of storage, as shown in Figure 2. The necessity of desiccated storage was also demonstrated, as assay fidelity dropped off significantly when stored without desiccant pellets.

To determine whether paper-based immunoassays, after stabilization and storage, could detect MMP-9 in saliva samples rather than in buffer only, saliva was spiked with MMP-9 across the clinically relevant concentration range and tested. The composition of the solution applied to the assay was 99.8−

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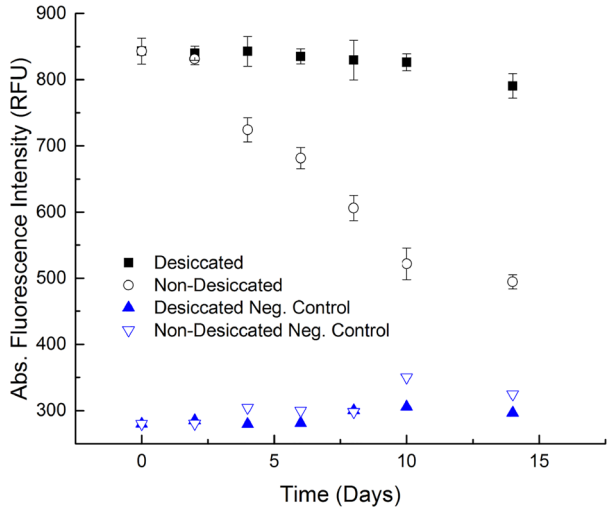

Figure 2. Stability of capture antibodies in paper MMP-9 assay. Detection of 4.3 nM MMP-9 demonstrated that stabilized assays stored with desiccant pellets in a zip-lock bag maintained similar signals to those stored for 0 days.

99.9% saliva; 0.1−0.2% was PBS in which the antigen aliquots were stored. To minimize the clogging effect of highly viscous mucins in saliva, saliva spiked with antigen was frozen to precipitate mucins, thawed, and centrifuged, and the supernatant was used in the paper immunoassays. This procedure produced approximately 0.75 mL of useable, mucin-removed saliva for every 1 mL produced by a person, meaning patients need only produce less than 15 μL of saliva to perform the assay.

The efficacy of this method in reducing saliva viscosity and nonuniform flow patterns was demonstrated in a test where (A) untreated, (B) centrifuged, and (C) frozen, thawed, and centrifuged saliva was flowed across 1-cm-wide strips of Whatman 1 chromatography paper, and the fluid front was tracked using food dye (Figure 3). It was apparent that the flow rate uniformity and viscosity of the saliva improved dramatically with the freezing/centrifugation treatment, and the impact of a freezing step was demonstrated.

As shown in Figure 4, the stabilized and stored immunoassays effectively detected MMP-9 in saliva across the clinically relevant range. Assay results for both MMP-8 and MMP-9 indicate that the freezing-centrifugation technique did not significantly affect antigen binding ability or concentration in the final supernatant, nor did storage reduce assay performance, as colorimetric readouts with treated saliva on stored assays were not lower in intensity than readouts from assays performed with known concentrations of antigen added to buffer.

A periodontal disease biomarker assay with a quantifiable colorimetric readout allows for more accurate identification of at-risk patients and disease stage—two shortcomings of current periodontal disease diagnostic methods. The low end of the relevant clinical concentration range assessed in these assays indicates gingivitis, where clinicians recommend hygiene-related interventions, whereas the medium and higher concentration levels in these ranges indicate mild to severe chronic periodontal disease. Depending on the stage of disease, various medications or surgeries may be necessary.[24]

Dental offices in developed countries are the near-term clinical settings targeted by this work. To make this device and methodology useable in very low resource areas, where a centrifuge may not be available, a hand-powered, low-cost paper centrifuge, such as the one described by Prakash and co-workers,[25] could be used to separate out proteinaceous molecules. Zhang et al. also demonstrated the efficacy of fiberglass pads in separating viscous elements from human saliva for use in lateral flow paper assays.[13] The use of either technique could greatly decrease the technological needs of the paper immunoassay described here.

In conclusion, to improve reagent stability and saliva compatibility of cellulose-based immunoassays, we developed an antibody stabilization technique, using the addition of trehalose and subsequent drying in a vacuum oven at 45 °C, to render capture antibodies preapplied to the paper surface able to detect MMP-8 and -9 in human saliva. Assays produced a brightly visible colorimetric readout across the full clinically relevant concentration range for MMP-9. Future work will include testing the device on patient saliva samples, adapting the assay into a more automated, user-friendly platforms, such as the 3D vertical flow devices used by Schonhorn et al.,[26] and examining stability of all assay reagents. It is likely that assay time can also be reduced upon integration into an automated platform.

Until now, analysis of saliva using cellulose-based, vertical flow immunoassays had not been reported, and while various methods have been used to reduce the viscosity of saliva in commercialized and lab-based saliva diagnostic tests,[13,14,27] the question of whether saliva processing steps reduce the concentration of protein biomarkers or their ability to bind with antibodies had not been addressed. These improvements further progress toward clinically available periodontal disease diagnostics, but also demonstrate potential for increased useability of many other paper-based immunoassays. Salivary protein biomarkers of a wide range of diseases, such as oral squamous cell carcinoma, lung cancer, Alzheimer's disease, Huntington's disease, and pulmonary tuberculosis have been identified,[28−32] and the availability of low-cost diagnostic technologies that can process saliva and be preprepared and stored until needed could greatly expand global diagnosis and treatment of these diseases.

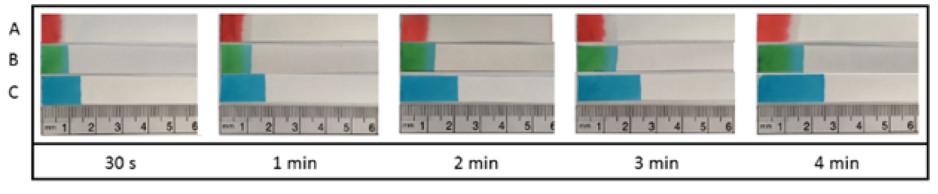

Figure 3. Flow profile at various time points of whole saliva (A) untreated, (B) centrifuged and supernatant extracted, and (C) frozen, thawed, and centrifuged and the supernatant extracted.

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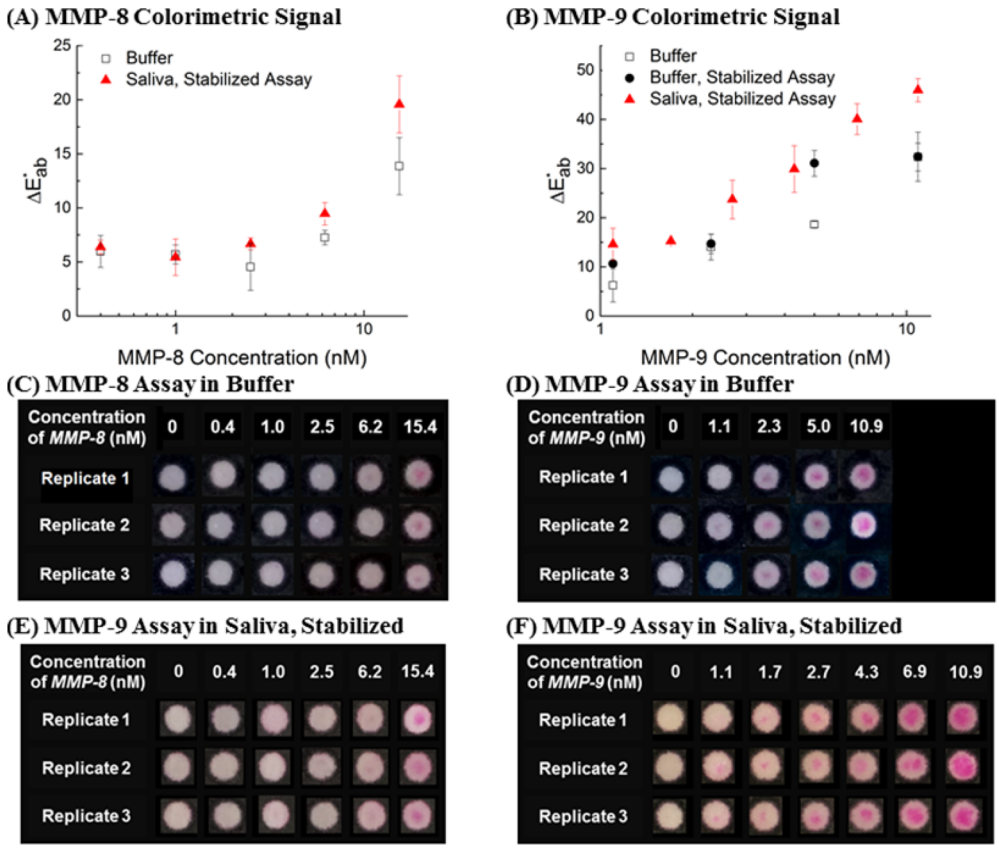

Figure 4. Colorimetric readout of MMP-8 and -9 assays measured as perceived color difference between antigen-containing samples and negative control tests (A,B), and visualized colored readout (C−F). Colorimetric signal of nonstabilized assays detecting MMP-8 and -9 spiked in buffer was comparable to that of stabilized and stored assays used to detect MMP-8 and -9 in human saliva.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.7b00745.

Detailed materials and methods; supplementary data referenced throughout text (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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The Supplementary Information includes a more detailed explanation and schematic of the MMP-8 and -9 sandwich immunoassays and polymerization-based signal amplification reaction used in the main manuscript, as well as the protocols used for preparing aldehyde-functionalized chromatography paper and conjugating eosin to streptavidin as a signal amplification reporter molecule. Also included is experimental data that supports the need for stabilization of MMP capture antibodies, as opposed to simply letting reagents dry on a benchtop, as well as demonstration that the stabilization method chosen in the study preserves MMP capture antibody functionality through 14 days of storage. A comparison is presented of the effects of lyophilized vs. vacuum oven dried treatments on the colorimetric readout of MMP-9 assays. Lastly, the protocol that was developed for reducing saliva viscosity is also included.

Supplementary Information:

1) Materials and Methods

Whatman No. 1 chromatography paper, lyophilized bovine serum albumin (BSA), and glycerol were purchased from VWR (Radnor, PA, USA). Potassium periodate (KIO4), poly(ethylene glycol) diacrylate (Mn=575) (PEGDA), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (VP), eosin Y disodium salt, dimethyl sulfoxide (DMSO), 10X phosphate buffered saline (PBS), phenolphthalein, hydrogen chloride, and Tween® 20 were obtained from Sigma Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) and sodium chloride were purchased from Avantor Performance Materials (Center Valley, PA, USA). Eosin-5-isothiocyanate (EITC) was obtained from Marker Gene Technology (Eugene, OR, USA). Streptavidin was obtained from Rockland Immunochemicals Inc. UltraCruzTM Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Recombinant human matrix metalloproteinase-8 (MMP-8) and MMP-9 protein, anti-MMP-8 and -9 antibodies (capture and biotinylated reporter) were obtained from R&D Systems (Minneapolis, MN, USA). 3A Molecular Sieve was obtained from Sorbent Media (Spring, TX, USA).

2) Schematic of Immunoassay and Polymerization Amplification Reaction

The immunoassay to detect MMP-8 and MMP-9, which was performed in these experiments is described in the section following this. A visual diagram of the reaction is shown below.

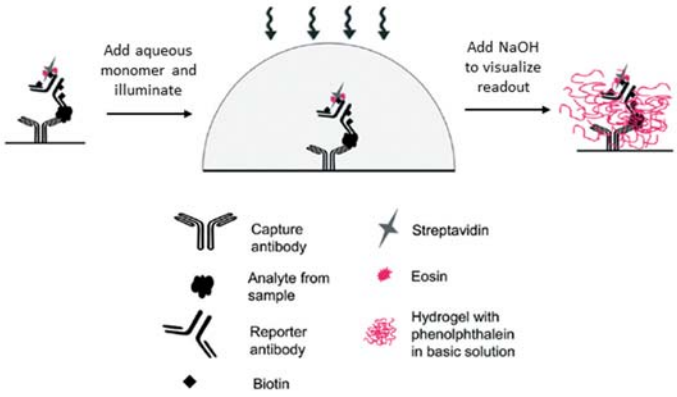

Figure S1: Schematic of sandwich immunoassay and polymerization-based signal amplification reaction. Adapted from Lathwal, S.; Sikes, H. D. *Lab Chip*, **2016**, *16*, 1374-1382. DOI: 10.1039/c6lc00058d. Copyright 2016 Hadley Sikes.

3) Sandwich Immunoassay Protocol

A Xerox ColorQube solid ink printer was used to pattern oxidized chromatography paper with hydrophobic ink to define hydrophilic, circular wells as test zones[1,2]. The wells were washed with deionized water, and 0.5 mg/mL capture antibody in 10% v/v glycerol in 1X phosphate buffered saline (PBS) was applied to the wells. The strips were incubated overnight in a humid chamber. Excess liquid was blotted, and the wells were washed with PBS. The strips were blocked by applying 1X tris-buffered saline and incubated for 1 hour. The strips were washed and samples, either with or without target antigen, were applied to the hydrophilic test zones, 10 μL per well. For calculating the limit of detection and the efficacy of stabilization methods, antigen was dissolved in 1% v/v bovine serum albumin (BSA) in PBS. Negative controls contained only the solution that the target antigen was dissolved in (either 1% v/v BSA in PBS or saliva for the assays described here). After incubation for 1 hour and washing, 5 uL of 50 μg/mL biotinylated reporter antibody was added to each well and incubated for 1 hour. After PBS washing, 10 μL of 0.2 μM eosin-conjugated streptavidin was added to each well and incubated for 30 minutes. Wells were blotted and washed sequentially with 0.1% Tween 20 in PBS, PBS, and de-ionized water. Strips were allowed to dry for 15 minutes in a dark drawer before fluorescence imaging to estimate the concentration of eosin bound via the antibody-antigen sandwich assay. Strips could be stored for months before visualizing the presence of target antigen with a polymerization reaction.

4) Colorimetric Detection via Polymerization Reaction

An amplification solution (103 uL poly(ethylene glycol) diacrylate, 20 uL triethanolamine, 10.4 uL 1-vinyl-2-pyrrolidinone, 200 uL phenolphthalein (0.25 wt% in 50 vol% ethanol in water), 20 uL 1N HCl, 0.3 μM eosin Y, and water) was made fresh. 20 μL was applied to negative control chromatography paper and illuminated with 30 mW/cm2, 522 nm light supplied by an ampliPHOX reader to determine the bulk polymerization time. At this time, regardless of antigen presence, a colored polymer forms. An exposure time just below the bulk polymerization time (t_{assay}) was determined. A drop of monomer solution was applied to each reaction well of the processed immunoassay strips and exposed to light for t_{assay}. A drop of sodium hydroxide was added after light exposure to generate a bright, pink signal from any polymer hydrogels that had

formed in the test zone. A picture of the colorimetric signal was taken using a cell phone camera and the signal intensity was analyzed using ImageJ.

5) Preparation of Aldehyde-Activated Paper

Functionalization of chromatography paper for immunoassays has been described previously[1]. 3" × 8" sheets of Whatman No. 1 chromatography paper were soaked in a 0.03 M KIO_4 solution at 65°C for 2 hours. After the reaction, the sheets were washed three times by dipping them in fresh deionized water for one minute each and pouring off the water at the end. After the last wash had been poured out, the sheets were blotted with paper towels and dried in a desiccator for at least 12 hours. A wax mask containing circular wax-free regions (3 mm in diameter) was printed on the dry sheets using a solid ink printer set to the default parameters for photo-quality printing. The printed sheets were placed in an oven (150 °C) for 90 seconds. As a result of the heat, the wax melted and spread through the thickness of the paper and created circular (2 mm in diameter) hydrophilic test zones separated by hydrophobic wax barriers. The presence of the aldehyde groups in the test zones allowed us to covalently immobilize amine-containing molecules (such as the anti-MMP-9 capture antibody in this study) to the surface of the paper through a Schiff-base linkage. The presence of the aldehyde groups in the test zones was confirmed by adding 2 μL of 2,4-dinitrophenylhydrazine and observing the change in color from yellow to orange. The sheets of aldehyde-functionalized paper were stored in a desiccator until use.

6) Conjugating EITC to Streptavidin:

The method of conjugation of EITC to proteins has been described previously[1-4]. EITC (1 mg) was dissolved in 100 μL of DMSO to prepare a 10 mg/mL EITC stock solution. To prepare the streptavidin conjugate, 10 μL of the EITC stock solution was mixed with a 100 μL solution of streptavidin (1 mg/mL) in 0.1 M sodium bicarbonate buffer (pH 9.7) to give a total reaction volume of 110 μL and the reaction mixture was protected from light and placed at 4 °C overnight. During the reactions, the isothiocyanate functional group of EITC reacts with the amine group of the lysine residues of the proteins to form a thiourea bond. At the end of the reaction, the excess EITC was separated from the eosin-conjugated streptavidin by size-

exclusion chromatography with Sephadex matrix (Micro G-25 Spin-Column). UV–visible absorbance spectroscopy was used to determine the concentration of the protein and the average number of eosin molecules coupled to each streptavidin molecule by taking an absorbance scan of the purified conjugate (Figure S2), and using the following equation.

$$\frac{n_{EITC}}{n_{SA}} = \left(Abs_{525}/\varepsilon_{EITC,525}\right)/\left[\left\{Abs_{280} - \left(Abs_{525}\varepsilon_{EITC,280}/\varepsilon_{EITC,525}\right)\right\}/\varepsilon_{SA,280}\right]$$

Where n_{EITC} is the number of molecules of eosin, n_{SA} is the number of molecules of streptavidin, Abs_{280} and Abs_{525} are the measured absorbance values at 280 nm and 525 nm, respectively, $\varepsilon_{EITC,280}$ is 26,800 $M^{-1}cm^{-1}$, $\varepsilon_{SA,280}$ is 173,000 $M^{-1}cm^{-1}$, and $\varepsilon_{EITC,525}$ is 90,200 $M^{-1}cm^{-1}$.

The purified and characterized conjugates were diluted to make 50% v/v glycerol stock and stored at -20 °C until use.

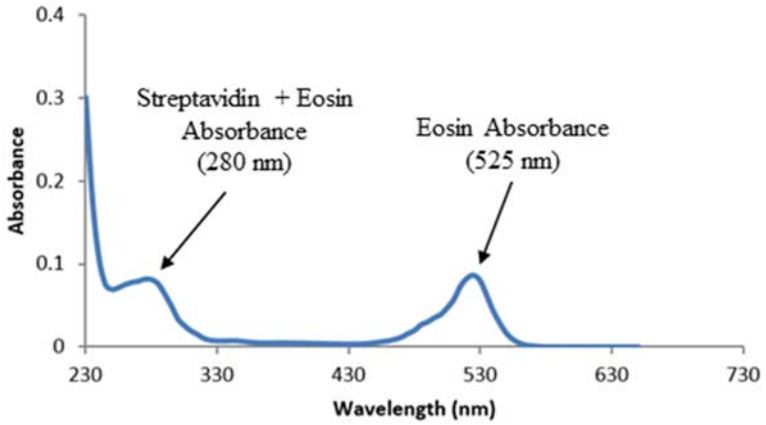

Figure S2: UV-vis absorbance spectrum for purified streptavidin-EITC conjugate

7) Image Acquisition and Analysis

Fluorescence images to detect surface-immobilized eosin species were acquired using an Olympus IX-81 microscope with a Texas Red filter set (Ex. 560/55, Em. 645/75). The colorimetric readout after polymerization was recorded using a MotoE (2nd generation), running Android 5.0.2, smartphone camera for Figure 1A and 1B. An iPhone SE camera was used to

record the signals shown in Figure 4. Images compared to one another (MMP-8 vs. MMP-9 biomarker readout, stabilization treatment techniques) were always acquired using the same camera. Because ambient lighting and operator positioning affect image color[5], those variables were controlled in the setup. The smartphone was held stationary in a clamp a set distance directly above the test well to be imaged. Lighting conditions were controlled by using a constant imaging area illuminated by a desk lamp, with overhead light blocked and diffused using a sheet of white printer paper. To quantify colorimetric readout, images of reaction wells were converted to L*a*b* color space, a 3-dimensional color space designed to approximate human vision and color perception[5]. The difference between the negative controls and the wells with a colorimetric polymer readout due to antigen binding was calculated with ΔE*ab, a CIE metric of the perceived difference between two colors. ΔE*ab was calculated using the formula $\sqrt{(L^* - L^*{}_0)^2 + (a^* - a^*{}_0)^2 + (b^* - b^*{}_0)^2}$, where $L^*{}_0$, $a^*{}_0$, and $b^*{}_0$ and L*, a*, and b* are the coordinates of the negative control and test sample, respectively.

Images for Figure 3 were also obtained using an iPhone SE camera. All image analysis, such as ΔE*ab and fluorescence intensity calculations, were performed using ImageJ, and in all cases, the entire circular well (hydrophilic zone contacted with sample) was analyzed.

8) Demonstration of Need for Stabilization of Paper-based Immunoassay

Two paper immunoassays were prepared with capture antibody, and blocked. One was left out to dry on the benchtop, uncovered and exposed to ambient pressure and temperature. The other was not allowed to dry. Both assays were then contacted with samples containing 10.9 nM (1000 ng/mL) MMP-9 in 1% BSA in PBS, and one negative control sample with no antigen. Reporter antibody and streptavidin-EITC were applied, and the final immunoassay was washed and visualized with a fluorescence microscope. The fluorescence intensity, indicating the binding success of the assay, was measured for both immunoassay. Both the dried and continuously treated assays had three replicates.

Abs. Fluorescence Intensity (RFU)	*Replicate #1 (1000ng/mL)*	*Replicate #2 (1000ng/mL)*	*Replicate #3 (1000ng/mL)*
Dried at Ambient	156	148	152
Continuously Treated	230	205	212

Table S1: Absolute fluorescence intensity of paper-based immunoassays wet and continuously treated, and immunoassays dried at ambient conditions after pre-sample treatment.

A two-sample, equal variance T-test was performed on the two sets of data. The two-tailed critical t-value was 2.78 and the t-value for the data was 8.14, indicating that the null hypothesis must be rejected, and there is a statistically significant difference between the samples (p=0.001).

9) Comparison of Stabilization Treatment Methods for Anti-MMP-9 Capture Antibodies on Paper

Several stabilization techniques were investigated: lyophilization, drying in vacuum oven at 45°C, drying at 4°C, and drying in a desiccator. For all tests, 4 uL of a 5% w/v solution of trehalose in 1X PBS was applied to wells that had been modified with capture antibodies. Following a 10 minute incubation, the trehalose solution was blotted to remove excess, 2 µL of trehalose was applied again, and the paper strips were subjected to one of the various drying treatments. After drying treatment, the strips were stored in a desiccator for two to fourteen days. It was found that lyophilization and vacuum oven drying best allowed capture antibodies to maintain functionality. To determine an optimal stabilization technique, lyophilization or vacuum oven dried paper assays were compared in their ability to detect MMP-9 in samples across the clinically relevant range.

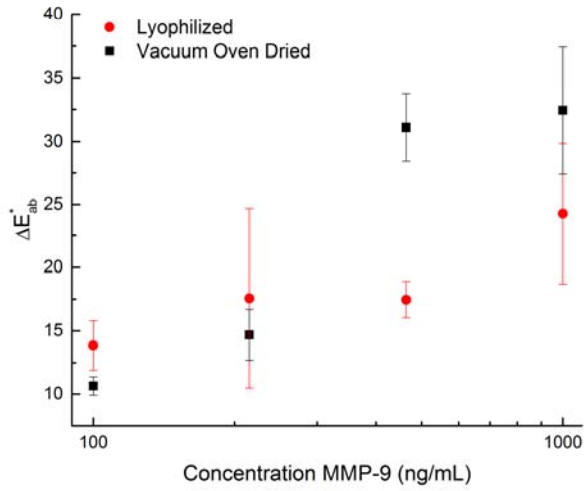

Figure S3: For immunoassays that were treated via lyophilization and vacuum oven drying, ΔE^*_{ab} was calculated for the colorimetric readout for various concentrations of MMP-9 compared to a negative control. The perceived colorimetric difference between positive and negative controls—and thus the clearness of the assay—was greater for paper devices pre-treated in a vacuum oven.

At low antigen concentrations, both techniques produce similar colorimetric contrast (ΔE^*_{ab}). However, at high antigen concentrations, vacuum oven drying treatment gave between 1.3 and 1.8 times greater *L*a*b** visibility than lyophilized test strips, indicating the former treatment better preserved antibody fidelity.

10) Comparison of Stabilized and Stored Paper-based Immunoassay vs. Wet, Continuously Treated Paper-based Immunoassay

Two paper immunoassays were prepared with capture antibody, and blocked. One was left stabilized with 5% trehalose in PBS and dried in a vacuum oven, as described in the Experimental section, and stored in a desiccator for 14 days. The other was prepared with capture antibody and blocked on day 14, and was not allowed to dry. Both assays were then contacted with samples containing 10.9 nM (1000 ng/mL) MMP-9, and one negative control sample with no antigen. Reporter antibody and streptavidin-EITC were applied, and the final immunoassay was washed and visualized with a fluorescence microscope. The fluorescence

intensity, indicating the binding success of the assay, was measured for both immunoassay. Both the stabilized and continuously treated assays had three replicates.

Abs. Fluorescence Intensity (RFU)	*Replicate #1 (1000ng/mL)*	*Replicate #2 (1000ng/mL)*	*Replicate #3 (1000ng/mL)*
Stabilized w/ trehalose; dried in vacuum oven; stored 14 days in desiccator	218	216	202
Continuously Treated	226	229	249

Table S2: Absolute fluorescence intensity of paper-based immunoassays wet and continuously treated, and immunoassays stabilized and stored for 14 days after pre-sample treatment.

A two-sample, equal variance T-test was performed on the two sets of data. The two-tailed critical t-value was 2.78 and the t-value for the data was 2.49, indicating that the null hypothesis holds, and there is *no* statistically significant difference between the samples ($p=0.07$).
Assay stability was also tested by storing stabilized assays in a ziplock bag with 3A Molecular Sieve zeolite pellets. Assays that had been stabilized with trehalose addition and vacuum oven drying were placed in two quart-sized ziplock bags, one containing 25g of molecular sieve, and placed in a laboratory bench drawer for 0-14 days. At intervals, assays were removed and used to detect 4.3 nM (400 ng/mL) of MMP-9 in 1% BSA in PBS buffer. This antigen concentration is in the dynamic range for MMP-9, and was expected to show the effects of storage on the assays more clearly than tests to detect 10.9 nM of MMP-9. As seen in Figure 2, the assay readout was not significantly different after 14 days from the readout after 0 days of storage. The necessity of desiccated storage was also demonstrated, as assay fidelty dropped off significantly without storage with zeolite.

11) Protocol for Saliva Sample Preparation Pre-Assay:

Saliva was used to dilute a 0.44 mg/mL stock solution of MMP-9 in 1X PBS to 100-1000 ng/mL MMP-9, and the MMP-9 spiked saliva was frozen overnight at -20°C. It was thawed and centrifuged at 5000 rpm for 25 minutes. The supernatant was extracted and applied to paper assay wells. Negative controls were pure saliva supernatant.

Optimal incubation times for the saliva samples and number of washes between reagent application were tested in a factorial design experiment by incubating for 1 and 2 hours, with double washes of 1X PBS (4x20 uL PBS) and the same wash used for serum immunoassays (2x20 uL PBS). Three replicates were tested for each combination of washes and incubation time. 50 ug/mL of mouse IgG in PBS was applied to the aldehyde-activated paper immunoassay base and incubated overnight. The surfaces were blocked with 1X TBS, washed, and 50 ug/mL biotinylated anti-mouse IgG in saliva was applied for 1 hour or 2 hours. For negative controls, only treated saliva was applied. After washing, 0.1 uM streptavidin-EITC in 1% BSA in PBS was applied for 30 min.

The fluorescence intensity indicating eosin concentration bound to the surface was measured for each test. The results are summarized below. It was found that incubating the treated saliva samples for 2 hours did not improve the positive readout intensity. Increasing the number of washes reduced signal for both the negative and positive control, as to be expected, but it reduced the positive signal more than the negative signal. Because a user's perception of a positive colorimetric signal depends strongly on the perceived color difference between the positive and negative readouts (ΔE^*_{ab} in CIELAB coordinates was used here), the difference between the concentration of eosin on negative and positive tests is very important to the clearness of positive readouts. For this reason, the 2x20 uL PBS washes were deemed a better procedure.

Abs. Fluorescence Intensity (AU)	*1 hr-1X washes*	*2 hr-1X washes*	*1 hr-2X washes*	*2 hr-2X washes*
50 ug/mL IgG	808	890	637	695
0 ug/mL IgG	436	362	301	321

Table S3: Absolute fluorescence intensity of paper with mouse IgG, biotinylated anti-mouse IgG in saliva, and streptavidin-EITC applied sequentially. Fluorescence intensities for 50 ug/mL samples are the average over 3 replicates. Standard deviations are 58, 26, 84, and 68, respectively.

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