Point-of-Need Disposable ELISA System for COVID-19 Serology Testing

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Abstract

A disposable enzyme-linked immunosorbent assay (dELISA) device for at-home or doctor's office use was developed to detect SARS-CoV-2 antibodies. Serology testing for SARS-CoV-2 antibodies is currently run using well-plate ELISAs in centralized laboratories. However, the scale of serology testing needed for epidemiological and clinical screening studies will overwhelm existing clinical laboratory resources. Instead, a point-of-need device that can be used at home or in doctor's offices for COVID-19 serology testing must be developed and is one of four target products prioritized by the World Health Organization. Lateral flow assays are common and easy to use, but lack the sensitivity needed to reliably detect SARS-CoV-2 antibodies in clinical samples. This work describes a disposable ELISA device that is as simple to use as a lateral flow assay, but as sensitive as a well-plate ELISA. The device utilizes capillary-driven flow channels made of transparency films and double-sided adhesive combined with paper pumps to drive flow. The geometry of the channels and storage pads enables automated sequential washing and reagent addition steps with two simple end-user steps. An enzyme label is used to produce a colorimetric signal instead of a nanoparticle label in order to amplify signal and increase sensitivity, while the integrated washing steps decrease false positives and increase reproducibility. Naked-eye detection can be used for qualitative results or a smartphone camera for quantitative analysis. The device can detect antibodies at 2.8 ng/mL from whole blood, which was very close the concentration of detectable target in a well-plate ELISA (1.2 ng/mL). In this study the dELISA system was used to detect SARS-CoV-2 antibodies, but we believe that the device represents a fundamental step forward in point-of-care technology that will enable sensitive detection of many other analytes outside of a centralized laboratory.

1. Introduction

SARS-CoV-2, the causative agent of COVID-19, has infected at least 32 M people and caused the deaths of over 980,000 worldwide (September 25, 2020).[1] The SARS-CoV-2 virus represents a dire threat to public health and the global economy. SARS-CoV-2 is highly infectious, making isolation one of the most effective methods of slowing disease spread. As societies attempt to return to normal, the question of how to determine who can leave isolation and return to work safely must be addressed. This question

is difficult to answer because it is unclear who has been infected and recovered and is therefore immune, given the high prevalence of asymptomatic individuals. Serology assays that detect the presence of anti-SARS-CoV-2 antibodies can be used to help answer these questions.[2]

To detect active infections, the approved SARS-CoV-2 detection methods rely on nucleic acid amplification technologies like PCR and LAMP to detect viral RNA.[3] While these methods are sensitive and specific, they are also expensive and complicated. The Medicare reimbursement cost for the CDC approved RT-qPCR assay is $100 and many labs report that this rate is insufficient.[4] More importantly, nucleic acid methods detect active infections and cannot determine who has been infected previously. Serological assays that detect antibodies are required to determine prior infection status and are traditionally done in laboratory settings using enzyme-linked immunosorbent assays (ELISA) or at the point of care using lateral flow assays (LFAs). These assays can also determine candidates for convalescent plasma donation, an increasingly important treatment strategy.[2] While traditional ELISAs have superior analytical performance, they require expensive equipment and samples must be shipped to a centralized laboratory for testing. The volume of serology tests needed in the US alone for a robust serosurvey of different populations will overwhelm clinical laboratory resources and samples may take days or weeks to process. Additionally, at ~$60/sample the cost of a test would be a significant financial burden to individuals, insurance companies and/or governments.[5] The World Health Organization has outlined four key target product profiles in the effort to fight the pandemic, and one is a point of care serology assay that takes < 20 min to perform, can be used by an untrained person, and costs < $12.[6] Traditional LFAs meet these requirements, but are lacking the analytical performance needed for such critically important testing.

LFAs use a series of porous membranes to wick sample across a detection zone. Target analyte is captured on a nitrocellulose membrane using a capture agent and is typically labeled with nanoparticles that form a colored line for naked-eye detection.[7] LFAs can be performed in a matter of minutes, require no external instrumentation, cost ~$5 per device, and can be used outside of a centralized laboratory. Several LFAs have been developed to detect SARS-CoV-2 antibodies but results from these devices are not trusted in large scale surveys because of poor clinical sensitivity and specificity.[8] Commercially available LFAs for SARS-CoV-2 serology testing report sensitivities of 80-96% and specificities of 80-99%, which are lower than the desired sensitivities and specificities outlined by the WHO (>95% and >99% respectively).[9-11] Further, in a study comparing six commercial rapid LFAs to an ELISA for COVID-19 serology testing, the ELISA outperformed all six LFAs for clinical sensitivity and specificity.[12] In that study the most sensitive LFA reported a sensitivity of 60% while the ELISA reported 79% sensitivity for the same samples. LFAs will inherently perform more poorly than ELISAs for two reasons: 1) They cannot wash excess reagent and/or sample from the detection zone to mitigate non-specific adsorption, and 2) nanoparticle labels cannot amplify colorimetric signal like enzymatic reporters. Previous attempts to create simplified ELISAs for use at the point-of-need have been successful, but require multiple timed steps,[13, 14] have inefficient washing,[15] are inconsistent because of end-user intervention, and can take over one hour,[16] limiting their practicality in at-home settings.[13, 17-19] There is a pressing need for a SARS-CoV-2 serology test that is as easy to use as a one-step LFA with the sensitivity and specificity of an ELISA.

This article describes a disposable ELISA system (dELISA) that automatically performs sequential washing and reagent addition steps using capillary-driven flow in hydrophilic channels and paper pumps for colorimetric detection of SARS-CoV-2 antibodies. The dELISA uses a recently developed pump-free microfluidic device to achieve automated and sequential sample transport, plasma separation from whole-blood, reagent delivery, and washing steps.[20] Individual steps of the assay are controlled through simple but highly effective valving and flow control structures in a laminate device with capillary-driven channels for fluid transport previously reported by our group.[20, 21] The channel geometry in the laminate device enables programmable flow of sample, washing buffer, and reagents for sequential delivery to a detection zone on a nitrocellulose membrane. These processes are performed with only two end-user steps: sample addition followed by buffer addition. Sequential flow of reagents and washing buffer stands in contrast to an LFA where all steps occur within the sample matrix without washing. The device is made of a combination of PET films, pressure sensitive adhesive, and nitrocellulose. As a result, devices are inexpensive (~$1) and can be made in large quantities. This manuscript discusses the flow control methods embedded in the dELISA along with the colorimetric assay that was designed specifically for use in the device. Anti-SARS-CoV-2 IgG nucleocapsid protein (anti-N protein) was detected using the N protein as a capture probe and a horseradish peroxidase secondary antibody (HRP-Ab) as the label. The chromogenic substrate 3,3'-Diaminobenzidine (DAB) was used for colorimetric detection. The performance of the disposable ELISA assay was compared to standard well-plate ELISA and antibodies were detected from whole blood, a common matrix for serology testing. Although the application of the device in this manuscript is serology testing for Covid-19, the technology will open the possibility of at-home ELISA testing for many more targets.

2. Materials and Methods

2.1 Device Construction

The fluidic channels in the device are constructed from 3M™ 9962 polyester film, which is 99 µm thick and coated with a proprietary hydrophilic coating on both sides. Four layers of the 9962 film are cut using a CO_2 laser cutter (Epilog, Zing 1000) to form channels for flow. These layers were designed in CorelDRAW X4. 3M™ MP467 double-sided adhesive is used between each piece of 9962 film to bind the film together and to form a gap between the layers. Each piece of double-sided adhesive is 50 µm thick and are patterned with the same CO_2 laser cutter. Three layers of double-sided adhesive are laminated between the layers of 9962 film to create the final device as shown in Figure 1. Layers are shown in more detail in Figure S1. 3×5 mm^2 glass fiber pads (Millipore Sigma, GXDX203000) were used as conjugate release membranes and the secondary antibody and substrate were dried and stored on these pads. The pads are inserted into the device before the final 9962 film layer is sealed on top. A plasma separation membrane (Vivid GX Membrane, Pall Corporation) was integrated above the main channel between the buffer inlet and the nitrocellulose. Finally, a 3×15 mm^2 nitrocellulose membrane (GE FF120) striped with capture antigen is inserted into the end of the channel and a waste pad made of GE CF4 membrane is placed at the end of the nitrocellulose. To demonstrate sequential delivery and washing in the device tartrazine (yellow dye, 1870 µM) and erioglaucine (blue dye, 800 µM) were used.[21]

2.2 Anti-SARS-CoV-2 Assay

The anti-SARS-CoV-2 immunoassay used SARS-CoV-2 nucleocapsid protein (N protein AA133-419) as the capture antigen. Recombinant SARS-CoV-2 N protein was produced as previously described.[22] Briefly, a bacteria-codon optimized gBlock was cloned into a pET28a bacterial expression with a C-terminal 6xHis tag. Recombinant protein was expressed in BL21(DE3) pLysS *E. coli* and purified by nickel affinity and size exclusion chromatography in 50 mM HEPES buffer (pH 7.4) and 500mM NaCl throughout purification to reduce aggregation. Protein purity and quality was verified by SDS-PAGE gel electrophoresis. N protein was striped onto the nitrocellulose strip with a reagent dispenser (Claremont Bio). The striping solution contained 45 mM trehalose, 4.5% glycerol, and 0.5 mg/mL N protein. The trehalose and glycerol were used to improve storage capability. Roughly 120 ng of N protein was added to each 3 mm nitrocellulose strip. The secondary antibody was an anti-mouse-IgG conjugated to horse radish peroxidase (HRP) (Abcam ab97040, Lot no. 3327554). The antibody was diluted to 5 µg/mL in a solution of 0.01 M FeSO4-EDTA, 4% Trehalose, and 0.1% BSA to improve long term storage.[23] Two 5 µL aliquots of the secondary antibody solution (50 ng) were sequentially dried onto a 3x5 glass fiber pad. The colorimetric substrate used was 3,3'-Diaminobenzidine (DAB). Pierce™ DAB Substrate kit from ThermoFisher, which included a 10x solution of DAB and a peroxide buffer, was used for the substrate and washing buffer. 15µL of the 10x DAB solution was added to a 3x5 glass fiber pad in three 5µL aliquots
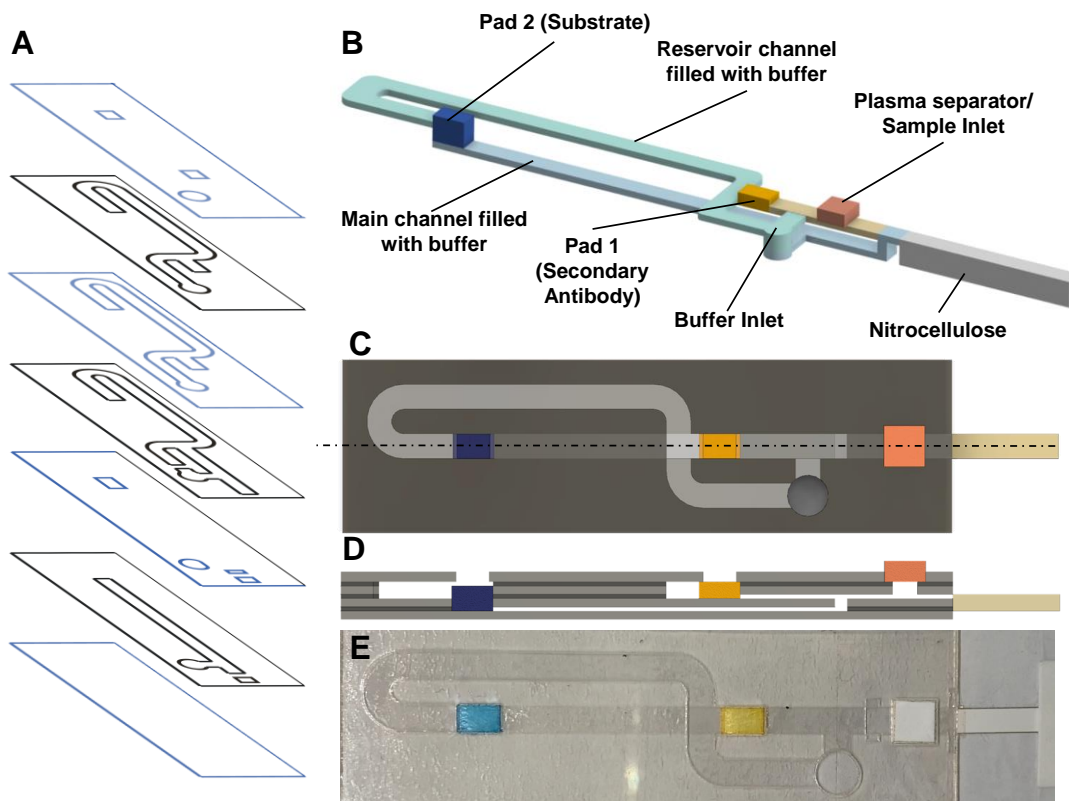

Figure 1. A) Designs for each layer of the device. Blue layers are transparency sheets and black layers are double-sided adhesive B) Three-dimensional representation of the channels and reagent pads within the device. C) Top view device schematic. D) Cross-sectional view of device cut along the dashed line in C. E) Top view of a real device.

and air dried at 37ºC for 20 min. The peroxide buffer was adjusted to a pH of 6.5 with sodium hydroxide and Tween-80 (Fisher Scientific) was added to a concentration of 0.1%.

To start the assay, 10 µL of sample is added to the Vivid plasma separation membrane (sample pad). The target analyte used was SARS-CoV-2 nucleocapsid antibody (GeneTex Cat. No. GTX632269 Lot No. 43936). The target antibody was diluted in phosphate buffer pH 7.2 or single donor human whole blood (Innovative Research) to concentrations ranging from 0.01 ng/mL to 100 µg/mL. After the sample is added to the sample pad, 75 µL of wash buffer is added to the buffer inlet (Figure 1). Once buffer has been added, the waste pad pumps the buffer through the system for ~20 min at which point the nitrocellulose membrane is imaged using a Motorola One smartphone. The color intensity at the test line is quantified using NIH ImageJ. Here, the image of the test line is converted to grayscale and inverted. The grayscale intensity across the test line is divided by the intensity adjacent to the test line to obtain a "gray ratio" for quantification. The ratio is used to reduce any error caused by variation in lighting. Lighting for this project was also stabilized with a 16 LED light box for consistency.[24, 25]

2.3 Well-plate ELISA

The analytical performance of the dELISA was compared to a traditional well-plate ELISA using the same capture antigen, target, and secondary antibody. In the well-plate ELISA, polystyrene plates were coated with 50 µL of 0.2 µg/mL N protein overnight on a shaker at 4°C and then blocked with 4% dry non-fat milk powder in 1X PBS and 0.1% Tween-20 at RT. 50 µL of the target antibody solution (GeneTex Cat. No. GTX632269 Lot No. 43936) was incubated in the wells for 1 h at RT on a shaker followed by washing 3X with 0.1%Tween-20 in PBS (200 µL). The secondary antibody is the same used in the dELISA (Abcam ab97040), and 50 µL of 0.1 µg/mL was incubated in each well for 1 h. The wells were washed 3X before addition of the 50 µL 1-step Ultra TMB substrate (Thermo Fisher Scientific, 34028). After 4 min the reaction was quenched with 1 M H_2SO_4 and absorbance readings were taken with a Perkin Elmer Victor X5 plate reader at 450 nm.

3. Results and Discussion

3.1 Automated sequential flow design

The dELISA contains laminate flow channels for programmable reagent release and washing that generates sequential flow, a nitrocellulose membrane strip where the sandwich immunoassay is performed, and a waste pad to drive flow (Figure 1a). Importantly, the flow channel was designed in a multi-layered format to achieve sequential delivery of the sample, buffer, and reagents to the nitrocellulose membrane after two simple end-user steps. The assay steps are as follows: 1) Sample addition by user; 2) buffer addition by user; 3) sample is washed through nitrocellulose membrane with washing buffer via capillary-driven flow and target is conjugated to proteins on detection zone; 4) rehydrated secondary antibody flows through the nitrocellulose membrane strip and conjugates to captured target 5) wash buffer removes excess secondary antibody from the nitrocellulose membrane strip; 6) rehydrated substrate flows through the nitrocellulose membrane strip, reacts with enzymatic label and produces a visible-by-eye color change.

The flow channel is divided into two sections: the main channel (lower) and the reservoir channel (upper). The sample and reagent inlets were placed in order of delivery to the detection zone along the

main straight channel (Figure 1b). The sample is added to the main channel through the plasma separation membrane while the reagents dried in the glass pad are rehydrated with buffer for delivery through the main channel. The timing of the sample and reagent delivery was controlled by adjusting the distance between the nitrocellulose and each reagent inlet. The opening above the glass fiber pads prevents air bubbles from forming in the main channel and helps maintain consistent flow. The reservoir channel, which connects upstream of each reagent pad, supplies the buffer solution to rehydrate reagents and generate flow in the main channel. Although the buffer channels are connected upstream of the glass pads, the buffer inlet is placed downstream of the glass pads so the buffer can fill both the main channel and the reservoir channel upon buffer addition. This channel geometry also makes the device more compact. Since the two reagent channels have different vertical positions, buffer can fill the main channel without being affected by the glass pads (Figure 1c). Finally, a burst valve was implemented under the separation membrane, which releases the sample and buffer into the main channel at the same time without air bubbles.[21] The burst valve initiates flow in a channel only after two separate channels meet to ensure proper flow timing.

To visualize these automated sequential steps, food dye was used in place of immunoassay reagents, (Figure 2). In addition to the still images in Figure 2b, a video showing the flow is included in the supplementary information. Here, blue dye is added to the sample pad, yellow dye is dried on glass pad 1 to simulate the secondary antibody, and blue dye is dried on glass pad 2 to simulate the substrate. After sample and buffer addition (steps 1 and 2) all channels are filled via capillary action, and the sample, secondary antibody, and substrate are connected via buffer. Once the channels are filled, the nitrocellulose strip and waste pad pumps the sample, buffer, and immunoassay reagents through the device. Because the
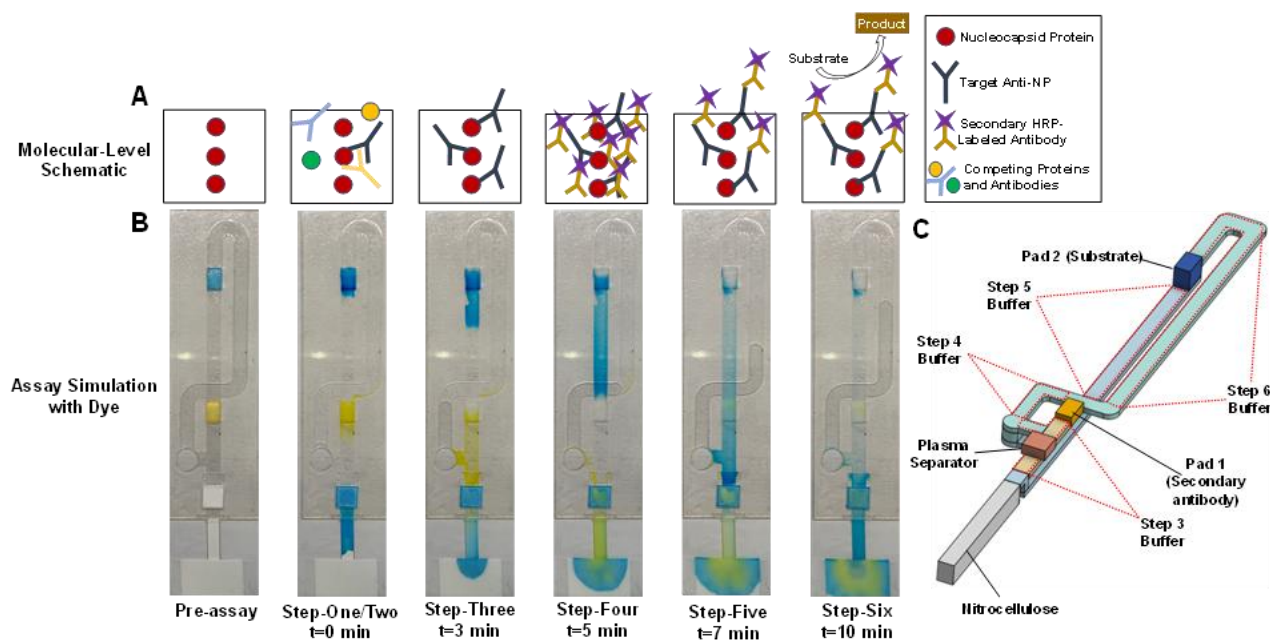

Figure 2. A) Molecular level representation of detection zone during different stages of the assay. B) Simulation of assay steps in the device using blue dye to mimic the sample and substrate, and yellow to mimic the secondary antibody. C) Three-dimensional representation of the channels in the device and how the reagents and buffer in the channels are used in different stages of the assay.

sample is placed at the front of the device it is programmed to pass through the nitrocellulose membrane first and the target is captured on the test line. Next, the buffer between the sample inlet and pad 1 (yellow) will flow through the nitrocellulose membrane and wash away excess sample constituents that might interfere with the remaining assay (step 3). The pressure difference from pad one to the nitrocellulose membrane is larger than that of the difference between the pad two (blue) and the nitrocellulose membrane, so the rehydrated enzyme label from the yellow pad flows through next and any target analyte on the test line will capture the enzyme label (step 4). Once the buffer above pad one is depleted, the flow from the blue pad to the nitrocellulose begins. The rehydrated substrate stored on pad two will be preceded by a slug of buffer between pads 1 and 2 in the main channel (step 5). Once the buffer has washed away excess label, the substrate reaches the test line and reacts with the enzyme label to produce a visible color change (step 6). After flow stops (~20 min) the color change is detected with the naked eye for qualitative detection, or imaged (smartphone camera) for quantitative information. The volume of buffer used to wash the sample or reagents through the nitrocellulose membrane can be controlled by changing the length of channels for any given assay. In the current device design, the total volume of washing buffer was 75 µL and the assay takes 20 min to run.

3.2 Anti-SARS-CoV-2 Assay Optimization

Once sequential reagent delivery and washing steps were confirmed, an immunoassay for anti-SARS-CoV-2 antibodies was performed using the dELISA device. Separate glass fiber pads were used to store the secondary antibody and substrate. A drying buffer (section 2.2) was used to dilute the secondary antibody to improve storage capabilities.[23] The assay parameters mentioned in section 2.2 were optimized, and the key results for washing buffer pH, capture antigen concentration, secondary antibody concentration, and DAB concentration are shown in Figure 3 with a blue star indicating the conditions chosen for the final assay. One of the struggles of running a sandwich immunoassay in the dELISA vs. a well-plate ELISA is that assay conditions must be constant instead of optimized for each individual sample addition or washing step. For example, a commercial
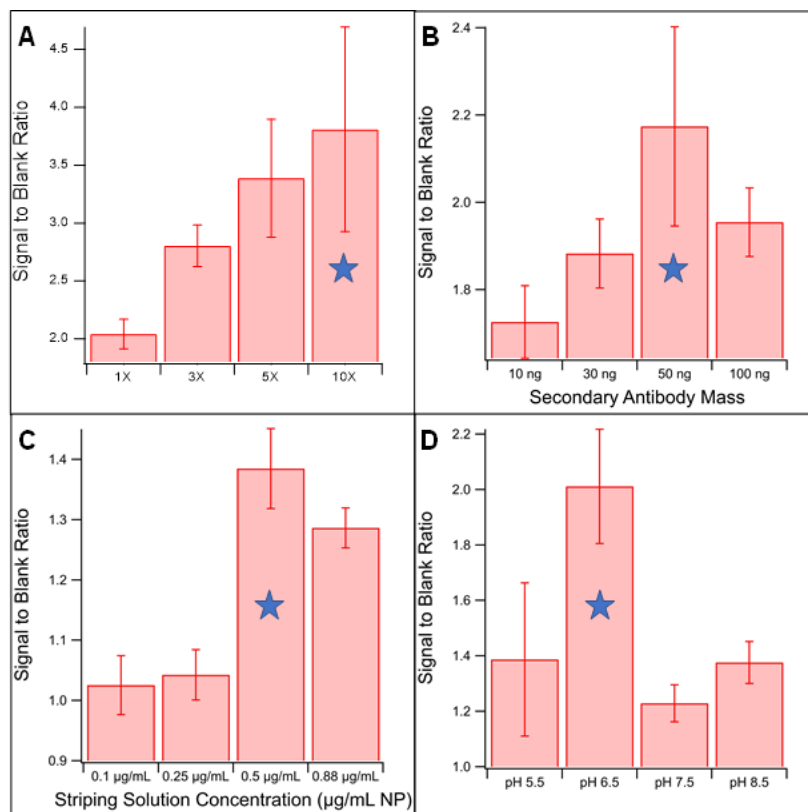

Figure 3: Optimization results for (A) Commercial DAB solution concentration dried on pad 2; (B) mass of secondary antibody dried on pad 1; (C) concentration of striping solution; (D) pH of washing buffer. Star represents the condition used for final assay

peroxide buffer was used as the washing buffer to improve the activity of HRP with DAB, but the buffer was adjusted from a pH of 5.5 to 6.5 for the final assay. While changing from 5.5 to 6.5 decreased the HRP activity, it improved the antibody-antigen binding, which had a larger impact on assay sensitivity. 1 µg/mL target antibody was used during the optimization experiments as that concentration falls within the linear range of the assay.

3.3 *Assay from Whole-Blood*

Whole blood was used as the sample matrix because plasma from whole blood is the most common matrix for serology testing. In an at-home setting blood could be obtained with a lancet to prick a finger and draw a drop of blood, similar to diabetic glucose testing. To ensure that red blood cells do not interfere with the colorimetric readout on the nitrocellulose membrane, they must be removed using a plasma separation membrane. In the dELISA device, the membrane is integrated just upstream of the nitrocellulose membrane (Fig. 1). After adding 10 µL of sample to the plasma separation membrane, red blood cells are captured in the membrane and plasma flows into the device and through the nitrocellulose. Video S2 in the supplementary information captures this process along with the rest of the assay. A dose-response curve was generated using blood spiked with anti-N protein antibody (Figure 4a). The data was fit to a four-parameter logistic curve as is common for sandwich immunoassays (Equation S1).[26] The detection limit using the dELISA in blood was 2.8 ng/mL, which is sensitive enough to detect anti-N protein IgG from clinical samples (>10 µg/mL).[27] The detection limit was
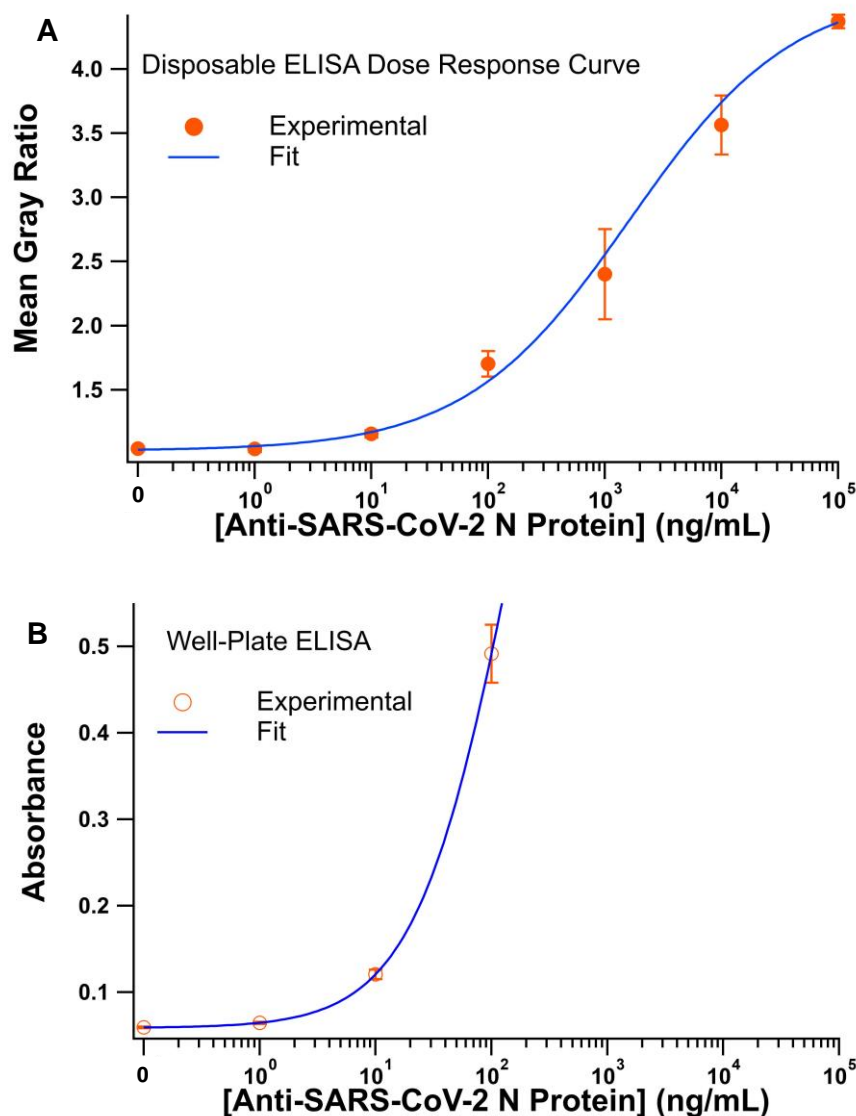

Figure 4. A) Dose-response curve of anti-N protein spiked into whole human blood using the dELISA. B) Dose-response curve of anti-N protein obtained with a well-plate ELISA. The data for both curves was fit to a 4-parameter logistic curve

calculated using Eq S2. Future studies will focus on using the dELISA with real patient samples to establish a clinical sensitivity and specificity for the device.

In upcoming clinical studies, the results will be validated with a well-plate ELISA, so in this work results were also compared to a well-plate ELISA using the same antigen and secondary antibody. The results for the well-plate ELISA are shown in Figure 4b. The well-plate ELISA dose-response curve was once again fit with a 4-parameter logistic curve and a detection limit of 1.2 ng/mL was calculated. The detection limit of the dELISA (2.8 ng/mL) is roughly the same as the detection limit in the dELISA (1.2 ng/mL). These results exceeded expectations for the dELISA considering that a well-plate ELISA uses absorbance values from a plate reader, while our device quantifies results using images from a smartphone. The advantages of the dELISA over a traditional well-plate assay include faster assay time (2.5 h versus 20 min), decreased end-user steps (13 vs 2), increased portability, decreased cost, and increased ease of use. Hands-on time and preparation time are also significantly less in the dELISA than the well-plate ELISA. Most importantly, the dELISA is a true at-home test that can be run outside of a centralized laboratory by an untrained end-user. These advantages are common for POC assays but are typically offset by sacrificing analytical performance. This is not the case for the dELISA, which has the sensitivity of a well-plate ELISA and is as easy to use as an LFA.

The question of whether the dELISA could be used to detect Anti-SARS-CoV-2 antibodies from real blood or nasopharyngeal samples is yet to be answered, but the detection limits presented here are promising as well-plate ELISAs have been used to detect actual SARS-CoV-2 antibodies types at higher concentrations.[27] Well-plate ELISAs and even LFAs have detected lower concentrations of antibodies previously, but we believe the limiting factor in the current dELISA setup is the capture antigen and secondary antibody and their specificity toward the particular commercial Anti-NP used. To improve results further a thorough screening of suitable secondary antibody for clinical anti-NP similar to the work presented by Cate et al. would need to be conducted.[28] Additionally, the drying procedure for the secondary antibody could be improved through lyophilization, and increasing the sample volume would increase sensitivity, but also increase assay time.

4. Conclusion and Future Directions.

This article describes a system that enables ELISA-level performance in a disposable and easy-to-use device. The dELISA was used to detected anti-SARS-CoV-2 antibodies from whole blood with a detection limit of 2.8 ng/mL, which is nearly the same as a traditional well-plate ELISA (1.2 ng/mL). The dELISA can achieve such sensitive results with a colorimetric read-out because an enzyme label is used to amplify the signal and sequential washing and reagent addition steps are performed automatically after sample and buffer addition. Qualitative detection can be performed with the naked eye, or a smartphone can be used to capture quantitative information. Future iterations of the device will use electrochemical sensing as a more quantitative alternative to colorimetric detection. Amid the current pandemic, next steps will focus on improving the assay for more robust serology testing that includes IgM, IgA and IgG antibodies, and simplifying use by integrating a housing and blister pouches for buffer storage. We will also design an assay for soluble nucleocapsid protein from nasal swabs so an active infection can be identified as well as a previous infection. Eventually, we envision this technology will be used in resource-limited settings to detect analytes such as influenza, malaria, or other viral proteins, biomarkers for chronic

illness such as heart failure or cancer, and/or other antibodies at levels that were previously undetectable outside a centralized laboratory.

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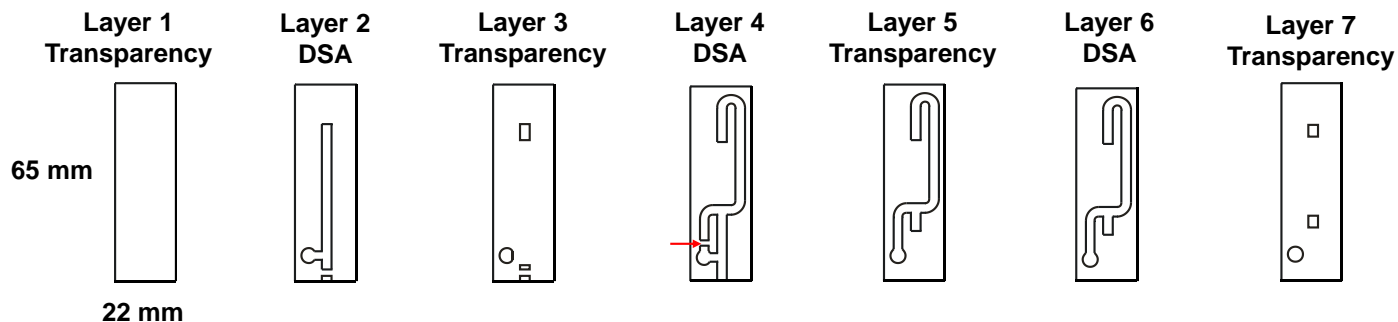
Supplementary Information

Figure S1: Design of each layer of the dELISA. Layers are currently assembled by hand with the aid of a cold-press laminator. During assembly, the marked section on layer 4 of DSA is cut out to complete the connection in the channel. The DSA material that is cut is included initially to hold all layers together.

Equation S1: 4-Parameter Logistic model used to fit the data from the dose-response curve. Y is the signal, x is the target concentration, a is the expected response (Y) at x=0, b is the slope of the curve at point c, which is the target concentration that corresponding to Y=(a+d)/2, and d is the expected response when the target concentration is infinitely high (Assuming no hook effect).

$$Y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}}$$

Equation S2: The equation used to calculate the detection limit from the 4-parameter logistic fit. Parameters a, b, c, and d are defined in Equation S1 above. SD_b is the standard deviation of the blank and R_b is the response or signal of the blank.

$$LOD = c \times \left(\left(\frac{(a - d)}{(3 \cdot SD_b + R_b) - d}\right) - 1\right)^{\frac{1}{b}}$$