COVID-19 first appeared in December of 2019 in Wuhan, China. Since then it has become a global pandemic. A robust and scalable diagnostics strategy is crucial for containing and monitoring the pandemic. RT-PCR is a known, reliable method for COVID-19 diagnostics which can differentiate between SARS-CoV-2 and other viruses. However, PCR is location dependent, time consuming and relatively expensive. Thus, there is a need for a more flexible method which may be produced in an off-the-shelf format and distributed more widely. Paper-based immunoassays can fulfill this function. Here we present the first steps towards a paper-based test which can differentiate between different between the Spike protein of various coronaviruses, SARS-CoV-1, SARS-CoV-2 and CoV-HKU1 with negligible cross reactivity for HCoV-OC43 and HCoV-229E in a single assay which takes less than 30 minutes. Furthermore, our test can distinguish between fractions of the same Spike protein. This is done by an altered assay design with four test line locations where each antigen builds a unique, identifiable binding pattern. The effect of several factors, such as running media, immunoprobe concentration and antigen interference is considered. We find that running media has a significant effect on the final binding pattern where human saliva provides results while human serum leads to the lowest signal quality.
Developing a paper-based antigen assay to differentiate between coronaviruses and SARS-CoV-2 Spike variants

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ABSTRACT: COVID-19 first appeared in December of 2019 in Wuhan, China. Since then it has become a global pandemic. A robust and scalable diagnostics strategy is crucial for containing and monitoring the pandemic. RT-PCR is a known, reliable method for COVID-19 diagnostics which can differentiate between SARS-CoV-2 and other viruses. However, PCR is location dependent, time consuming and relatively expensive. Thus, there is a need for a more flexible method which may be produced in an off-the-shelf format and distributed more widely. Paper-based immunoassays can fulfill this function. Here we present the first steps towards a paper-based test which can differentiate between different coronaviruses, SARS-CoV-1, SARS-CoV-2 and CoV-HKU1 with negligible cross reactivity for HCoV-OC43 and HCoV-229E in a single assay which takes less than 30 minutes. Furthermore, our test can distinguish between fractions of the same Spike protein. This is done by an altered assay design with four test line locations where each antigen builds a unique, identifiable binding pattern. The effect of several factors, such as running media, immunoprobe concentration and antigen interference is considered. We find that running media has a significant effect on the final binding pattern where human saliva provides results while human serum leads to the lowest signal quality.

Introduction

The COVID-19 global pandemic which emerged in Wuhan, China in December of 2019 has infected millions of people and caused or contributed to the death of hundreds of thousands and led to an economic downturn. Diagnosis of COVID-19 using diagnostic tools is preferable over clinical symptoms due to the nonspecific nature of COVID-19 symptomology. The majority of patients suffer non-specific, mild symptoms mostly consisting of cough, fever, muscle pain and nausea. Additionally, an estimated 20 – 80% of patients are asymptomatic, with varying reports. Traditional laboratory diagnostics such as RT-PCR have high sensitivity and diagnostic accuracy, but are expensive and time-consuming, and may not be locally available. Paper-based immunoassays can complement PCR and contribute to a better diagnostics strategy. While they commonly suffer from relatively low selectivity and especially sensitivity, compared to PCR, paper-based tests are cheap to produce locally or in a at-scale manufacturing facility, can be made in an off-the-shelf format and stored for prolonged periods of time in mild conditions, e.g. room temperature or 4 °C. Furthermore, their flexible design allows simultaneous detection of multiple targets.

Paper-based tests for SARS-CoV-2 detection can be classified as antibody (or serological) tests, i.e. those which measure the host immune response, and antigen tests, i.e. those which bind to viral antigens. Antigen paper-based immunoassays typically target the Spike (S) or Nucleocapsid (N) proteins. The S protein decorates the outside of coronaviruses and enables their uptake into cells. Variances in S protein structure determine the cellular uptake pathway and affinity of the virus for the cell. SARS-CoV-2, similarly to SARS-CoV-1 and HCoV-NL63, targets the ACE2 receptor in the oral and nasal cavities as its primary root of infection. The S protein has been shown to be a primary driver of viral evolution, where mutations greatly impact virus infection rate and is considered to be the primary difference in reproductive number between clades.

A low-cost, rapid, and user-friendly platform which can differentiate is desirable. Distinguishing between respiratory viruses with similar clinical symptoms is especially important not only to provide timely treatment but also better assess patient risk. Here, we present the first step towards developing a paper-based sensor which can differentiate between S proteins from different coronaviruses as well as Spike protein variants from SARS-CoV-2. The test relies on a sandwich
immunoassay and antibody cross-reactivity for antigen specific test patterns. We used a set of six commercially available antibodies and seven commercially available S proteins to establish the viability of the strategy. The test was able to differentiate between all antigens, including SARS-CoV-2 Spike fragments. Test limit of detection (LOD) was in the 0.1 nM range, which may be sufficient for viral detection in more severe cases. We also studied the effect of running medium on test efficacy, bovine serum albumin (BSA) human serum (HS) and saliva.15

Results

Immunoprobe Screening

We chose spike protein as a target because it protrudes outside the viral particle and is responsible for receptor recognition. It is composed of two parts, S1 and S2, where the receptor binding domain (RBD) is in S1.16 We obtained S1 proteins for SARS-CoV-2, SARS-CoV-1 (SARS) and non-lethal coronaviruses CoV-HKU1 (HKU1), HCoV-OC43 (OC43) and HCoV-229E (229E) to study the selectivity of our nanoparticle-antibody conjugates (NP-Ab conjugates or immunoprobes). SARS-CoV-2 was termed COVID to avoid confusion (Table 1 and S1).

Table 1. Antigens, antibodies and NP-Ab conjugates used

| Antigen   | Virus       | Antibody | NP-Ab    | Virus       |
|-----------|-------------|----------|----------|-------------|
| COVID 1   | SARS-CoV-2  | αC1      | NP-αC1   | SARS-CoV-2  |
| COVID 2   | SARS-CoV-2  | αC2      | NP-αC2   | SARS-CoV-2  |
| COVID 3   | SARS-CoV-2  | αS1      | NP-αS1   | SARS-CoV-1  |
| SARS      | SARS-CoV-1  | αS2      | NP-αS2   | SARS-CoV-1  |
| 229E      | HCoV-229E   | αS3      | NP-αS3   | SARS-CoV-1  |
| OC43      | HCoV-OC43   | αH       | NP-αH    | CoV-HKU1    |
| HKU1      | CoV-HKU1    |          |          |             |

All antibodies used were commercially available. Six antibodies were evaluated for antigen binding and sandwich immunoassay formation (Table 1 and S2). Two were raised against SARS-CoV-2 (αC1-2), three for SARS (αS1-3), and one for CoV-HKU1 (αH). Gold NP-Ab conjugates were synthesized using literature methods.17,18 Briefly, each antibody was conjugated to the NPs by physisorption, and then thiolated PEG was added after conjugation to backfill open areas on the NP surface. Dynamic light scattering (DLS) and UV-Vis spectroscopy were used to determine colloidal stability and size dispersion in PBS. DLS was used to measure particle size in BSA, saliva and HS. DLS of NPs showed hydrodynamic diameters \(D_H\) of ~65 nm which increased ~85 nm after antibody conjugation, supporting conjugation (SI Figures S1, S2). The size further increased to 120 – 200 nm in media, possibly due to protein adsorption to the NP surface.

Antibody target and off-target binding were studied using antigen and sandwich dipstick assays. In the former case the antigens were immobilized on nitrocellulose following which the prepped strips were immersed in an immunoprobe dispersion (Figure 1a). In the latter case sandwich immunoassay formation, that is binding between the paper printed antibody, antigen and immunoprobe, was tested (Figure 1e). α-rabbit IgG Fc (α-Fc), was immobilized at the control area during both tests. A signal at the test line certified fluid flowed through the paper strip. Localized signal emerges on the strip due to immunoprobe accumulation (Figure 1a and e). NP-αS2 and NP-αS3 NPs were eliminated due to poor performance in initial tests (SI Figure S3).

After running, strips were left to dry overnight at room temperature after which they were scanned and analyzed via ImageJ.19 The resulting grayscale values were normalized to 100% and plotted as a heatmap for clarity (Figure 1b), where white represents low intensity (< 20% of maximum value) and green high. Averaged grayscale values are provided in the Supporting Information.

In antigen binding tests, NP-αC1 and NP-αC2 bound to both COVID and SARS antigens, but not other coronavirus antigens. NP-αS1 bound to its target antigen, SARS, and exhibited some cross reactivity for COVID 1 and OC43. Immunoprobe binding in saliva resulted in similar signal intensities, but were notably lower in human serum (HS) (Figure 1b, S4 and S5), which could be attributed to HS screening of the immunoprobe function.9

We evaluated the ability of the immunoprobes to form an immunological sandwich in a dipstick assay (Figure 1c).20 Sandwich formation relies on two reactions, immunoprobe-antigen and printed antibody-antigen binding. The choice of both antibodies is primary during test design, where antibody pairs (on the immunoprobe / printed on the paper) with varying affinity and selectivity for a target can be used to detect a wider variety of antigens and develop a binding pattern.21 In this initial study we screened the pairing of an antibody with itself, i.e., NP-αS1 run with immobilized αS1 (NP-αS1/αS1), NP-αC1 with αC1 (NP-αC1/αC1), NP-αC2 with αC1 (NP-αC2/αC1), and NP-αH with αH (NP-αH/αH). αC2, a monoclonal antibody, did not form a sandwich with itself.

NP-αC1/αC1 and NP-αC2/αC2 were able to successfully form sandwich immunoassays with both COVID and SARS antigens, as indicated by the color resulting at the test line (Figure 1d, S6 and S7). NP-αS1/αS1 was able to detect the SARS antigen, but not COVID antigens. The observed difference in behavior is potentially due to protein folding. NP-αC1/αC1 and NP-αC2/αC2 exhibited no cross reactivity for 229E, OC43, and HKU1, and NP-αH/αH exhibited signal only with HKU1.

Pairs were also tested in saliva and HS (Figure 1d), and the behavior was largely similar to BSA except for NP-αH/αH exhibiting some cross reactivity towards OC43 in saliva. Saliva resulted in similar intensities to BSA, but intensities were significantly lower in HS, highlighting the importance of the biological media. Generally, direct antigen binding had a higher signal and cross reactivity than sandwich formation (Figure 1b and d).
Cross reactivity was more commonly observed in saliva compared to the other media. This could be attributed to slower flow possibly due to surface tension, which results in longer residence time of the immunoprobe-antigen complex near the test areas, and increases binding probability. A compounding factor could be saliva composition, which has different surfactants or macromolecules and a lower pH (6-7) compared to BSA and HS (7.4). We expect another large contributor to be the relatively low protein concentration of saliva, which is > 99% water (wt/wt) and ~ 1 mg/mL protein compared to BSA (30 mg/mL) and HS (60 – 80 mg/mL). Test backgrounds in all three media were similar, suggesting low non-specific binding of the immunoprobes to the paper.

Antigen cross reactivity could be attributed to proximity in phylogeny. Cross reactivity between SARS and SARS-CoV-2 antibodies and antigens was expected due to ~82% sequence similarities, while the proximity between the RBD regions is ~73%. NP-αH bound to its target antigen HKU1, as well as OC43 presumably due to proximity in phylogeny. Antigen sequences were compared between each other and the Wuhan reference strain structure (accession number YP_009724390.1) using MUSCLE. Structural differences in MUSCLE can arise from both sequence length and content therefore it is expected that some structural dissimilarities arise our antigens being recombinant fractions of the S protein. HKU1 was most dissimilar shoring the most structural similarity with the Wuhan reference strain (20%) and the least with COVID 1 (10%). SARS had a ~50% structural similarity with COVID 1 and 2 and ~28% with COVID 3 and the Wuhan reference train. Similarity between the COVID antigens and compared with the Wuhan strain were 30 -50%. Comparison of the RBD of all COVID antigens revealed a 100% structural similarity.

Quantifying sandwich immunoassay performance in different media

Performance of the sandwich immunoassays was investigated by quantifying their limit of detection (LOD) and effective dissociation constant ($K_{D}^{Eff}$) for their target antigens. NP-αC1/αC1, NP-αC2/αC1, and NP-αH/αH, were titrated with COVID 1 and HKU1 antigens in BSA, HS and saliva. Test line intensities were measured and fit with a modified Langmuir equation. $K_{D}^{Eff}$ was obtained from the fitting. LOD was calculated separately, defined as the background signal + 3x the standard deviation of the background. LODs ranged from 0.1 to 0.17 nM while $K_{D}^{Eff}$ values were in the 10$^{-10}$ M range (Figure 2a-c, Table 1 and S3).

Changing the running media impacted performance. Compared to BSA, antibody pairs generally performed similarly or better in saliva and worse in HS (Figure 2a-c, Table 1). The LOD of NP-αC1/αC1 was lowest in BSA and 2X higher in saliva and 7x in HS (Figure 2a and Table 1). Titration curves of NP-αC2/αC1 were lowest in BSA, followed by saliva (3x), and significantly higher in HS (9x) (Figure 2b).

Between the two SARS-CoV-2 pairs, NP-αC1/αC1 exhibited comparable behavior, but marginally outperformed NP-αC2/αC1 in all media by ~2X. This is consistent with sandwich results where NP-αC1/αC1 which had a higher intensity with COVID 1 than NP-αC2/αC1. NP-αH/αH behavior changed the most with running media, where LOD and $K_{D}^{Eff}$ values varied by two orders of magnitude between saliva and HS. LODs were 0.07 nM in saliva and 0.54 nM in HS. $K_{D}^{Eff}$ values were 0.06 nM in saliva and 2.78 nM in HS (Table 1, Figure 2c, d).
Control of antigen detection through cross reactivity

We then investigated how the test could be used a multiplexed test to differentiate between SARS-CoV-2 antigens with similar structure and other coronavirus species, building on the ability to strategically use cross-reactive antibodies to differentiate between different antigens. We first studied the relevance of both binding events on sandwich formation and antigen detection. The signal of combinations of all three antigens (SARS, COVID 1 and HKU1), immunoprobes (NP-αC1, NP-αC2, NP-αH) and printed antibodies (αS1, αC1, αH) was measured (total of 27 combinations, Figure S9a). Experiments were repeated in triplicate in BSA at antigen concentrations of 1 X 10⁻³ mg/mL.

SARS was detectable only with the NP-αC1/αS1 and NP-αC2/αS1 pairs. COVID 1 was detectable only with the NP-αC1/αC1 and NP-αC2/αC1 pairs, and HKU1 was detectable only with the NP-αH/αH pair (SI Figure S9b and c). This further confirmed HKU1 was not cross reactive with the other coronavirus antibodies.

These results reaffirm the efficacy of the cross reactivity strategy, where only SARS was detected at immobilized αS1 and only COVID 1 at immobilized αC1. We attribute this to the lower affinity of cross reactive interactions, which was confirmed by off target antigen titrations (SI Figure S10). The NP-αC1/αC1 and NP-αS1/αC1 antibody couples were titrated with their “off target” antigens, being SARS and COVID 1, respectively (SI Figure S10 and Table S4 and S5). Both showed reduced signal in all media compared to their “on target” immunoprobe performance. There was no significant difference in LOD and K_DEff for NP-αC1/αC1 compared to its “on-target” COVID 1 titration (Table S4 and S5).

Our results suggest that detection of SARS and COVID 1 is more dependent on the printed antibody rather than the one conjugated to the AuNP. On average, there was an order of magnitude drop in “off target” interactions, compared to “on target ones”, e.g. NP-αC1/αS1 to NP-αC1/αC1 with SARS (SI Figure S9b-c). Overall these results suggest that cross reactivity can be used for generating patterns.

Multiplexed test

We designed multiplexed assay to differentiate between spike antigens of SARS, COVID 1, 2 and 3 and simultaneously detect spike from a non-lethal coronavirus, HKU1. The strip consisted of a control and four test areas at differently shaped locations (Figure 3a). Choice of location shape was to differentiate locations easier. Test area geometry did not have a measurable impact on signal quality. The antibodies immobilized at the test locations were (bottom to top) αS1 (square), αC2 (triangle), αC1 (octagon) and αH (oblong). A 1:1 volumetric mixture of NP-αC1 and NP-αH was used to detect all antigens.

Running the assay with the different antigens produced characteristic binding patterns at the four test areas. SARS and COVID antigens were detectable on all locations except the αH area (oblong) in saliva (Figure 3b). SARS resulted in signal mostly at the αS1 location, with lower signals at αC1 and αC2. COVID 1 exhibited the highest signal at αC2 and lower signal with αC1 and αS1 (Figure S3b). COVID 2 was only detectable at αC areas with comparable signal intensity at both locations (Figure 3b). As expected from its structure, COVID 3 produced signal mostly at the αC2 area. 229E and OC43 antigens in saliva yielded no significant signal, and HKU1 was observable only at the αH test area.
Again, signals were similar in BSA and saliva but were either reduced in intensity or lost in HS. For example, SARS could not be detected in HS, but display a characteristic pattern in BSA and saliva (Figure 3c, top left). HKU1 signal was strongest in BSA and weakest in HS, which was consistent with single strip results (Figures 1d, 2c). Cross reactivity was observed more often in saliva, where SARS was detectable at the αC2 location, COVID 2 was detectable at the αS1 location and OC43 was detectable at the αH location.

**Interference between multiple antigens in multiplexed test**

We ran antigen mixtures in our test to investigate binding interference (Figure 4a). When a mixture of SARS + HKU1 was run, intensity appeared at all four test areas, with higher intensity at αH and αS1. This pattern resembled the additive intensity pattern of SARS alone plus HKU1 alone (Figure 4b and SI Figure S13e, i, j). This suggested that the antigens did not compete with each other for forming immunoprobe pairs.

Results suggest that the binding pattern in the presence of multiple antigens is similar to mathematically adding the two independent patterns if the two antigens did not bind to the same immunoprobe, e.g. SARS 1 and HKU1 (Figure 4b, S13 and S14).

However, for antigens which bind to the same immunoprobe, the pattern depended on the affinity of the interaction. When a mixture of SARS1 + COVID 1 + HKU1 was run, intensity appeared at all four test areas, showing that the test was able to simultaneously detect SARS, COVID1 and HKU1. However, the intensity pattern did not resemble what would be anticipated from simply adding the intensities of the individually run antigens (Figure 4c). Since SARS and COVID 1 interact with NP-αC1 with a similar dissociation constant ($K_D^{eff}$ (SARS) = 0.86 nM, $K_D^{eff}$ (COVID 1) = 0.42 nM) the pattern of the mixture depended on the relative concentration of the two antigens. In this case, the antigen with the higher concentration would have a more pronounced pattern (SI Figure S12 and S13a, b, f and S14a, b, f). Similar effects were observed for a SARS, COVID 1 and 2 mixture ($K_D^{eff}$ (COVID 2) = 0.10 nM, SI Figure S12).

Some challenges arose with the multiplexed strip. Background gradients were more common in longer strips. To accommodate for this, we limited the strip to 5 test locations and modified the image analysis. We immobilized antibodies with higher affinity further up the strip so they would not deplete the immunoprobe/antigen complexes before they encountered lower affinity immobilized antibodies. An optimal immunoprobe or immunoprobe mixture concentration was determined to be 12-17% of the total sample volume (SI Figure S15).

By understanding and controlling these principles, it was possible to simultaneously detect and differentiate all three coronavirus antigens (Figure 4c, SI Figure S13 and S14).

**Discussion**

The paper-based immunoassay investigated here was able to differentiate spike antigens from different coronaviruses by building a binding pattern through the number, arrangement and specificity of printed antibodies. Differentiation between SARS-CoV-1, SARS-CoV-2 and HKU1 S1 proteins was simpler based on their patterns where SARS bound mostly to the NP-αC1/αS1 pair while HKU1 bound exclusively to NP-αH/αH. Negligible cross reactivity with 229E and OC43 was observed.
The ability to differentiate between SARS-CoV-2 antigens is especially interesting and could yield new information for patient samples. We applied our test to three recombinant S1 fragments, COVID 1 with a length of 681 amino acids (aa), COVID 2 with 461 aa and COVID 3 with 229. Considering the high similarity in sequence (SI Figure S8) the difference in binding pattern can be attributed to their size. Thus, the use of binding patterns may be able to differentiate between different Spike products in patient samples. This may help address some of the questions raised about patient viral shedding over time.\(^{28}\) Differentiation between Spike protein variants or fractions may be improved by increasing the specificity and number of antibodies printed on the nitrocellulose. It is unlikely that the test design would be able to differentiate between Spike proteins from different clades due to high sequence similarity (> 99%).

The ability to detect and differentiate between antigen from other coronaviruses and antigen mixtures should not be overlooked. Co-circulation of non-lethal coronaviruses, other respiratory viruses and SARS-CoV-2 has been reported.\(^{29,31}\) Co-infections between SARS-CoV-2 with other respiratory viruses are rare, reported in 3-20% of cases, including co-infections with non-lethal coronaviruses in about 0.1-5% of cases.\(^{32-33}\) However, rare a recent study in the UK suggests that co-infection of SARS-CoV-2 with influenza has been shown to increase risk for poor patient outcomes.\(^{29}\) Co-infection may also help explain reported abnormal viral shedding patterns and differences in patient outcomes, so a test that can simultaneously differentiate between common respiratory viruses would help address these questions.

The running media impacted antigen binding, where strips run in BSA and saliva had comparable results with a 15% variation in signal, lower than test-to-test variability. Running assays in HS resulted in a reduction or complete loss of signal, which could be attributed to protein screening effects.\(^{17,34}\) LODs for COVID antigens in BSA and saliva were in the 0.0125 nM range (0.03 to 0.56 nM). In comparison, others have reported similar performance with a LOD of 0.62 ng/ml (0.025 nM) for half strips for SARS-CoV-2 nucleocapsid (N) proteins.\(^{20,35}\) We caveat that the LOD measurement was based on purified antigens in solution and not the full virus capsid in biological fluids, and matrix effects in patient samples or differences in spike secondary structure could influence performance. We estimate that the average number of Spike proteins in sputum swabs may be in the pM range for most cases and nM range in more severe cases. This estimate is based on the average copies of RNA per mL of saliva\(^{36}\), and the average number of spike proteins on a single virus.\(^{37}\) We assume that every RNA copy corresponds to a virus, that each virus has 100 Spikes on its surface and there are no free Spike proteins. This is a rough estimate due to the assumptions made (SI Calculations section). While the concentration of spike protein in patient samples over time has still not been definitively quantified, it is anticipated that the LOD here may be too high for visual readouts for most patient samples.\(^{37-40}\)

The intensity in a paper-based sandwich immunoassay depends on multiple factors, such as antibody affinity, immunoprobe concentration, antibody coverage on the NP, its size, concentration of the immobilized capture antibody, and several others.\(^{9}\) Varying these parameters can improve signal intensity without external enhancement approaches. Commercial diagnostics have solved this with dedicated readers for colorimetric or fluorescent readouts (Sofia, Quidel Corp.). Surface enhanced Raman spectroscopy (SERS) nanotags,\(^{41-42}\) isotachophoresis,\(^{43}\) and photothermal heating\(^{44}\) have all been used successfully to increase signal, sometimes as high as 100-fold. Simply running the flow back and forth over multiple passes can increase signal 5-fold, and does not require additional readout instrumentation.\(^{7}\) Additionally, concentrating the antigen by sample preparation techniques or paperfluidic design could increase signal. Ultimately, this sensitivity gap could be solved by a combination of techniques.\(^{45}\)

Test variability was estimated through the standard deviation and relative standard deviation (RSD) from all tests (SI Figure S16), and was estimated to be 36%, which did not change significantly between media or antigen mixtures. Typically, lower signals and weaker antigen-antibody interactions resulted in higher RSDs.

Results presented here can be used towards the development of COVID-19 paper-based dipstick and lateral flow assays (LFAs), a rapid diagnostic format that has aided in disease management, quarantine, and surveillance. The low production cost and relative ease of use of paper rapid diagnostics makes them suitable for both local and large scale production, making it a potentially powerful off-the-shelf complement to RT-PCR, which could help elevate strain on local diagnostic facilities to meet the massive demand for tests. The self-contained nature of paper-based tests makes them attractive for remote or mobile locations.
ASSOCIATED CONTENT
Supporting Information contains secondary figures and tables providing context for the text, calculations used and methods section. This material is available free of charge via the Internet at http://pubs.acs.org.

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DRH designed and carried out the studies, and wrote and edited the manuscript, HR and JGM wrote and edited the manuscript, KHS designed the studies, wrote and edited the manuscript.

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ABBREVIATIONS
BSA, bovine serum albumin; DLS, dynamic light scattering; HS, human serum; LOD, limit of detection; NP, nanoparticle; RSD, relative standard deviation.

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SYNOPSIS TOC
Supporting Information

Developing a paper-based antigen assay to differentiate between coronaviruses and SARS-CoV-2 Spike variants

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| Antigen name | Virus          | Description                        | Source | Product Code |
|--------------|----------------|------------------------------------|--------|--------------|
| COVID 1      | SARS-CoV-2     | COVID-19 Spike, aa Val16-Arg685    | Sino Bio | 40591-V08H   |
| COVID 2      | SARS-CoV-2     | COVID-19 Spike, aa Arg319-Phe541   | Sino Bio | 40592-V08H   |
| COVID 3      | SARS-CoV-2     | COVID-19 Spike                     | BEI    | NR-52366     |
| SARS         | SARS-CoV-1     | SARS Spike, aa Met1-Arg667         | Sino Bio | 40150-V05H1  |
| 229E         | HCoV-229E      | HCoV-229E Spike, Cys16-Asn536     | Sino Bio | 40601-V08H   |
| OC43         | HCoV-OC43      | HCoV-OC43 Spike, Met1-Leu794      | Sino Bio | 40607-V08H1  |
| HKU1         | CoV-HKU1       | HCoV-HKU1, Met 1-Arg 760           | Sino Bio | 40021-V08H   |
Table S2. List of antibodies used.

| Antibody name | NP-Ab conj. name | Virus        | Clonality  | Specificity         | Source  | Product Code |
|---------------|------------------|--------------|------------|---------------------|---------|--------------|
| αC1           | NP- αC1          | SARS-CoV-2   | Monoclonal | COVID-19 Spike      | Sino Bio| 40150-R007   |
| αC2           | NP- αC2          | SARS-CoV-2   | Polyclonal | COVID-19 Spike RBD  | Sino Bio| 40592-T62    |
|               |                  |              |            | aa 319-541          |         |              |
| αS1           | NP- αS1          | SARS-CoV-1   | Polyclonal | SARS Spike          | Invitrogen| PA5-81793   |
| αS2           | NP- αS2          | SARS-CoV-1   | Monoclonal | SARS Spike aa 490-510 | BEI     | NR617        |
| αS3           | NP- αS3          | SARS-CoV-1   | Polyclonal | SARS Spike aa 19-35 | Invitrogen| PA1-41090   |
| αH            | NP- αH           | CoV-HKU1     | Polyclonal | COVID-19 Spike aa 1-760 | Sino Bio| 40021-T60    |
Figure S1. Size reproducibility. (a) NP-αC1, (b) NP-αC2, (c) NP-αH, (d) NP-αS1. Particle sizes averaged from a N of 16, 12, 14 and 8 independent immunoprobe synthesis, respectively. Averaged size distribution of AuNPs cores used (N=2) included in all panels to show diameter change with functionalization.
Figure S2. DLS data. Size distribution of (a) NP-αC1, (b) NP-αC2, (c) NP-αH, (d) NP-αS1 in different media.

Figure S3. COVID 2 antigen test additional data. (a) Example dipstick strips and (b) averaged grayscale data (N=3) from an COVID 2 antigen binding test for full immunoprobe roster in BSA.
Figure S4. Effect of media, additional data. Example dipsticks antigen binding tests conducted in all three running media.

|         | BSA  | SARS | COVID 1 | COVID 2 | COVID 3 | Z29E | OC43 | HKU1 |
|---------|------|------|---------|---------|---------|------|------|------|
| NPaS1   | 77±1 | 34±2 | 3±28    | 3±27    | 7±8     | 7±11 | 88±9 | 0±0  |
| NPaC1   | 71±2 | 58±23| 84±22   | 56±20   | 2±2     | 1±3  | 3±6  |      |
| NPaC2   | 76±2 | 93±23| 98±21   | 80±11   | 9±7     | 27±7 | 5±5  |      |
| NPaH    | 2±5  | 5±4  | 10±4    | 3±2     | 0±4     | 71±11| 102±15|      |

|         | Saliva | SARS | COVID 1 | COVID 2 | COVID 3 | Z29E | OC43 | HKU1 |
|---------|---------|------|---------|---------|---------|------|------|------|
| NPaS1   | 84±11   | 60±2  | 4±8     | -2      | 10±1   | 5±17 | 81±1 5±1 |
| NPaC1   | 72±13   | 61±3  | 8±0     | 73±3    | 22±2   | 5±15 | 3±4 -2±4    |
| NPaC2   | 89±13   | 80±2  | 6±0     | 73±1    | 69±1   | 7±8  | 3±1±2 0±22   |
| NPaH    | 7±6    | 5±7   | 4±5     | 7±7     | 5±2    | 68±1 1±1 99±1 1±1 |

Figure S5. Direct tests intensity data. Grayscale values and standard deviations for immunoprobe binding directly to antigens, shown in Figure 1b.
Figure S6. Effect of media, additional data. Example dipsticks sandwich immunoassays conducted in all three running media.

| BSA   | SARS | COVID 1 | COVID 2 | COVID 3 | Z29E | OC43 | HKU1 |
|-------|------|---------|---------|---------|------|------|------|
| NPαS1 | 31±5 | 0±1     | 0±1     | 0±0     | 0±1  | 0±1  | 0±1  |
| NPαC1 | 25±5 | 66±7    | 85±18   | 19±45   | 7±6  | 6±6  | 3±2  |
| NPαC2 | 20±4 | 41±33   | 89±20   | 66±40   | 3±4  | 3±5  | 1±1  |
| NPαH  | 0±1  | 1±1     | 0±0     | 0±0     | 1±1  | 11±2 | 81±5 |

| Saliva | SARS | COVID 1 | COVID 2 | COVID 3 | Z29E | OC43 | HKU1 |
|--------|------|---------|---------|---------|------|------|------|
| NPαS1  | 43±31| 1±1     | 0±1     | 2±3     | 0±2  | 0±0  | 1±1  |
| NPαC1  | 23±17| 60±25   | 66±25   | 36±19   | 7±15 | 7±18 | 9±9  |
| NPαC2  | 23±9 | 72±7    | 90±8    | 85±8    | 5±4  | 5±0  | 3±0  |
| NPαH   | 6±3  | 7±2     | 5±0     | 5±1     | 10±3 | 41±3 | 80±3 |

| HS     | SARS | COVID 1 | COVID 2 | COVID 3 | Z29E | OC43 | HKU1 |
|--------|------|---------|---------|---------|------|------|------|
| NPαS1  | 22±12| 1±1     | 2±1     | 4±5     | 2±2  | 0±2  | 0±2  |
| NPαC1  | 20±7 | 53±6    | 78±6    | 28±5    | 6±7  | 5±4  | 5±3  |
| NPαC2  | 16±4 | 38±4    | 94±4    | 51±8    | 4±4  | 3±3  | 3±2  |
| NPαH   | 8±3  | 5±3     | 8±3     | 4±5     | 1±5  | 9±1  | 54±4 |

Figure S7. Immunoprobe pairs intensity data. Grayscale and standard deviations for immunoprobe sandwich formation shown in Figure 1d.
Figure S8. Structure comparisons between HKU1, SARS, COVID 1, 2 and 3 antigens and the Wuhan reference strain comparing their a) full protein structure, b) the RBD region of COVID 1, 2, 3 and the Wuhan strain, and c) 681 amino acid sequence between COVID 1 and Wuhan reference strain. Green areas denote at least three identical amino acids. Sequence similarity and sequence comparisons were obtained from MUltiple Sequence Comparison by Log-Expectation (MUSCLE).

Table S3. A, $K_D^{\text{Eff}}$ and $R^2$ values of pairs for target antigens in BSA, saliva, and HS averaged from at least three titration curves compared to the fitting of an averaged curve.

|                  | BSA          |                  |                  |                  |                  |                  |                  |
|------------------|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | A            | $K_D^{\text{Eff}}$ (nM) | $R^2$ | A          | $K_D^{\text{Eff}}$ (nM) | $R^2$ | A          | $K_D^{\text{Eff}}$ (nM) | $R^2$ |
|                  | Averaged Individual Fittings |                  |                  |                  |                  |                  |                  |
| NP-αC1/αC1 with COVID 1 | 55 ± 15 | 0.62 ± 0.25 | 0.95 ± 0.02 | 36 ± 13 | 1.7 ± 1.6 | 0.86 ± 0.11 | 68 ± 20 | 0.68 ± 0.63 | 0.94 ± 0.05 |
| NP-αC2/αC1 with COVID 1 | 68 ± 11 | 2.8 ± 3.1 | 0.98 ± 0.01 | 25 ± 8 | 4.2 ± 6.5 | 0.82 ± 0.07 | 65 ± 14 | 0.71 ± 0.65 | 0.96 ± 0.02 |
| NP-αH/αH with HKU1 | 62 ± 9 | 0.11 ± 0.07 | 0.93 ± 0.03 | 82 ± 9 | 2.3 ± 0.7 | 0.98 ± 0.01 | 80 ± 6 | 0.08 ± 0.05 | 0.90 ± 0.08 |
|                  | Averaged Curve Fittings |                  |                  |                  |                  |                  |                  |
| NP-αC1/αC1 with COVID 1 | 55 | 0.64 | 0.99 | 39 | 0.95 | 0.98 | 64 | 0.42 | 0.97 |
| NP-αC2/αC1 with COVID 1 | 64 | 1.38 | 0.99 | 23 | 1.92 | 0.98 | 63 | 0.5 | 0.98 |
| NP-αH/αH with HKU1 | 60 | 0.18 | 0.97 | 82 | 2.24 | 0.98 | 80 | 0.06 | 0.91 |
Figure S9. Testing immunoprobe pairs with all antigens. a) Schematic representation of combinations antibody pairs used. b) Test line images of representative stirps. c) Average test area intensities in BSA. Intensities were of all strips within the figure are normalized for simplicity. Average is from at least three independent batches.

Figure S10. Titration curves of selected pairs. a) NP- αC1/αC1 run with SARS, b) NP- αS2/αC1 run with COVID 1, c) NP- αC1/αC1 run with COVID 2.
Figure S11. Table with grayscale values and standard deviations for individual antigen binding patterns as shown in Figure 3.

Table S4. A, $K_D^{eff}$ and $R^2$ values of pairs for additional target antigens in BSA, saliva, and HS averaged from at least three titration curves compared to the fitting of an averaged curve.

|          | BSA |        |        | HS |        |        | Saliva |
|----------|-----|--------|--------|----|--------|--------|--------|
|          | A   | $K_D^{eff}$ (nM) | $R^2$ | A  | $K_D^{eff}$ (nM) | $R^2$ | A  | $K_D^{eff}$ (nM) | $R^2$ |
| Summed Fitting |     |        |        |    |        |        |      |        |        |
| NP-αC1/αC1 with SARS | 30 ± 6 | 1.0 ± 1.3 | 0.86 ± 0.13 | 26 ± 0 | 2.0 ± 2.1 | 0.94 ± 0.03 | 43 ± 6 | 0.72 ± 0.39 | 0.95 ± 0.01 |
| NP-αS2/αC1 with COVID 1 | 17 | 0.67 | 0.88 | - | - | - | 17 ± 3 | 0.85 ± 0.26 | 0.7 ± 0.26 |
| NP-αC1/αC1 with COVID 2 | 48 ± 8 | 0.23 ± 0.09 | 0.89 ± 0.1 | | | | |
| Averaged Individual Fitting |     |        |        |    |        |        |      |        |        |
| NP-αC1/αC1 with SARS | 28 | 0.52 | 0.87 | 26 | 1.63 | 0.97 | 39 | 0.54 | 0.96 |
| NP-αS2/αC1 with COVID 1 | 9 | 0.76 | 0.89 | 2 | 9.55 | 0.11 | 17 | 0.86 | 0.84 |
| NP-αC1/αC1 with COVID 2 | 47 | 0.21 | 0.95 | | | | | |
Table S5. LOD and $K_d^{\text{Eff}}$ values of pairs for target antigens in BSA, saliva, and HS.

|                        | BSA                  |                      | Saliva               |                      | HS                   |                      |
|------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|                        | LOD (nM)             | $K_d^{\text{Eff}}$ (nM) | LOD (nM)             | $K_d^{\text{Eff}}$ (nM) | LOD (nM)             | $K_d^{\text{Eff}}$ (nM) |
| NP-αC1/αC1 with SARS   | -                    | 0.76                 | 3.59                 | 0.86                 | -                    | 9.55                 |
| NP-αS2/αC1 with COVID 1| 0.05                 | 0.52                 | 0.08                 | 0.54                 | 0.75                 | 1.63                 |
| NP-αC1/αC1 with COVID 2| -                    | -                    | 0.01                 | 0.10                 | -                    | -                    |

Figure S12. Example strips and averaged intensities for antigen mixture binding patterns obtained in mixtures with the same concentration or volume of antigen. Calculated antigen concentrations, LOD and $K_d^{\text{Eff}}$ are provided. Figure shows that when concentrations are equal SARS and COVID 1 are detected at a comparable rate, however, when the COVID 1 concentration is increased it outcompetes SARS. Relative signal fraction on the various test locations are displayed as a percentage.
Figure S13. Running of alternative paper design with antigen mixtures. a) SARS + COVID 1, b) SARS + COVID 2, c) SARS + 229E, d) SARS + OC43, e) SARS + HKU1, f) COVID 1 + COVID 2, g) COVID 1 + 229E, h) COVID 1 + OC43, i) COVID 1 + HKU1 and j) SARS + COVID 1 + HKU1. All areas are normalized to the highest intensity and are the average of at least three independent immunoprobe batches.

|                   | BSA | HS | Sal. | SARS + COVID 1 | BSA | HS | Sal. | SARS + COVID 2 | BSA | HS | Sal. | SARS + 229E | BSA | HS | Sal. | SARS + OC43 | BSA | HS | Sal. | SARS + HKU1 | BSA | HS | Sal. | SARS + ME1 | BSA | HS | Sal. |
|-------------------|-----|----|------|----------------|-----|----|------|----------------|-----|----|------|-------------|-----|----|------|-------------|-----|----|------|-------------|-----|----|------|-------------|-----|----|------|-------------|
| **aH**            | 1 ± 3 | 1 ± 1 | 3 ± 0  | aH 0 ± 2 | 0 ± 0 | 2 ± 2 | aH 1 ± 1 | 1 ± 1 | 3 ± 1 | aH 2 ± 4 | 5 ± 8 | 8 ± 3 | aH 60 ± 11  | 40 ± 33 | 64 ± 7  |
| **AC1**           | 62 ± 6  | 51 ± 17 | 54 ± 7  | AC1 40 ± 16 | 51 ± 5 | 43 ± 7  | AC1 21 ± 8 | 11 ± 9 | 25 ± 5  | AC1 24 ± 9 | 18 ± 11 | 24 ± 1  | AC1 21 ± 8  | 34 ± 10 | 29 ± 10 |
| **AC2**           | 33 ± 13 | 17 ± 14 | 27 ± 7  | AC2 71 ± 16 | 62 ± 3 | 53 ± 6  | AC2 18 ± 11 | 6 ± 6  | 18 ± 8  | AC2 16 ± 13 | 13 ± 13 | 28 ± 6  | AC2 11 ± 8  | 8 ± 8  | 22 ± 2  |
| **AS1**           | 21 ± 19 | 9 ± 4 | 35 ± 14  | AS1 10 ± 6 | 4 ± 6  | 30 ± 18 | AS1 45 ± 10 | 13 ± 20 | 44 ± 25 | AS1 48 ± 23 | 31 ± 30 | 65 ± 10 | AS1 41 ± 16 | 54 ± 15 | 64 ± 17 |
| **COVID 1 + COVID 2** | BSA | HS | Sal. | COVID 1 + 229E | BSA | HS | Sal. | COVID 1 + OC43 | BSA | HS | Sal. | COVID 1 + HKU1 | BSA | HS | Sal. | SARS + COVID 1 | BSA | HS | Sal. | SARS + ME1 | BSA | HS | Sal. |
| **aH**            | 1 ± 2 | 0 ± 1 | 0 ± 2  | aH 0 ± 2 | 1 ± 1 | 0 ± 1  | aH 2 ± 6 | 1 ± 2  | 5 ± 3  | aH 55 ± 9 | 28 ± 19 | 61 ± 4  | aH 63 ± 5  | 41 ± 20 | 64 ± 22 |
| **AC1**           | 54 ± 9  | 54 ± 20 | 42 ± 9  | AC1 69 ± 14 | 61 ± 12 | 62 ± 6  | AC1 69 ± 13 | 52 ± 12 | 56 ± 13 | AC1 57 ± 10 | 59 ± 12 | 58 ± 12 | AC1 57 ± 12 | 57 ± 13 | 74 ± 14 |
| **AC2**           | 72 ± 17 | 56 ± 5  | 66 ± 10  | AC2 27 ± 14 | 18 ± 5  | 32 ± 10 | AC2 31 ± 13 | 18 ± 5  | 36 ± 8  | AC2 20 ± 11 | 15 ± 10 | 35 ± 20 | AC2 28 ± 10 | 18 ± 15 | 25 ± 8  |
| **AS1**           | 9 ± 3  | 0 ± 4 | 17 ± 10  | AS1 1 ± 9 | 0 ± 3 | 12 ± 11 | AS1 6 ± 10 | 1 ± 3  | 16 ± 14 | AS1 5 ± 11 | 1 ± 1 | 9 ± 8  | AS1 17 ± 27 | 0 ± 1  | 13 ± 3  |
Figure S14. Example strips of alternative paper design with antigen mixtures. a) SARS + COVID 1, b) SARS + COVID 2, c) SARS + 229E, d) SARS + OC43, e) SARS + HKU1, f) COVID 1 + COVID 2, g) COVID 1 + 229E, h) COVID 1 + OC43, i) COVID 1 + HKU1 and j) SARS + COVID 1 + HKU1.
Figure S15. Distributions of intensity in the test strip. a) Averaged total intensity on all test areas of strip run with BSA with COVID 1 with b) immunoprobe distribution on the paper and c) example strips for NP-αC2. d) Averaged total intensity on all test areas of strip run with BSA spiked with SARS1, COVID 1 and HKU1, e) NP-Ab conjugate distribution on the paper and f) example strips are also provided. Data shows that test area signal plateaus around 12 and 17% V/V of immunoprobes. Higher immunoprobe concentration increases non-specific interactions and the amount of particles in the wick.
Figure S16. Histograms of standard deviation and relative standard deviation as a measure of variability in altered test designs. a) and b) show the averaged standard deviation (for all tests) and relative standard deviation (for tests with a grayscale > 20% of the highest grayscale value). Histograms of the standard deviation from immunoassays run in c) BSA, d) HS and e) saliva run with a single antigen. Histograms of standard deviations obtained from immunoassays run in f) BSA, g) HS and h) saliva with antigen mixtures. Standard deviations were obtained from averaging the grayscale values for between three and six independent particle batches.
Calculations

Langmuir fitting

Antigen titration data was fit to a Langmuir curve:

\[ y = A \times \frac{x}{K'_{D} + x} \]

Where \( y \) is the grayscale value, \( A \) is a coefficient obtained from fitting and is related to the maximum value of the grayscale value, \( x \) is the antigen concentration and \( K'_{D} \) is the effective dissociation constant.

Limit of detection, LOD

LOD the concentration at which signals exceeded the background signal + 3x the variability of the background signal. This was calculated by obtaining the threshold value from experiments as follows:

Threshold signal (TS) = Average background grayscale + 3x standard deviation of background grayscale

Background grayscale in this case is the measured signal at 0 mg/mL antigen concentration.

The LOD, defined as the antigen concentration (M) above which meaningful signals are observed was then calculated as:

\[ LOD = TS \times \frac{K'_{D}}{A - TS} \]

Where \( K'_{D} \) and \( A \) are defined above.

Spike concentration in patient samples

Spike concentration calculations were based on the number of viral RNA copies obtained from 1 and the estimated number of Spike proteins per virus from 2. In this case the calculation was:

\[ N (\text{Spike}) = N (\text{Virus}) \times C (\text{Spike/Virus}) \]

Where \( N (\text{Spike}) \) and \( N (\text{Virus}) \) is the number of Spikes and viruses per mL of sputum and the \( C (\text{Spike/Virus}) \) is the average concentration of Spike proteins on a single virus.

\[ C (\text{Spike in M}) = \frac{N (\text{Spike})}{N_{A} \times V} \]
Where \( N_A \) is Avogadro’s number or \( 6.02 \times 10^{23} \) and \( V \) is the volume equal to \( 10^{-3} \) L.

**Methods**

**Synthesis**

**Gold NP Synthesis**

47.2 g of MilliQ water was weighed into a glass bottle. 1.4 mL of 10 mg/mL HAuCl\(_4\) (Sigma, product code: 254169) was pipetted in after which the bottle was placed in a water bath resting on a reaction station. A magnetic stirrer was used to homogenise the solution fast enough so that there is a visible vortex. The water in the bath was brought to a boil and left to fully equilibrate for at least 15 minutes. After this time the lid of the bottle was carefully removed and 0.9 mL of 10 mg/mL sodium citrate, originally tribasic sodium citrate (Sigma, product code: S4641) were added under vigorous stirring. The lid was placed back on the bottle which was left to react for ~15 minutes. During this time the solution changed colour from a dim yellow, to almost black to wine red as particles formed. Final concentrations of HAuCl\(_4\) and sodium citrate were 0.83 mM and 0.62 mM, respectively.

**Immunoprobe synthesis**

Synthesis was done in accordance with the principles laid out in our previous work. \(^3\) The appropriate amount of as synthesised AuNPs were pipetted into a LoBind Eppendorf (Sigma, product code: Z66613). The choice of vessel was deliberate as other plastics led to NP sticking to the sides. 10 \( \mu \)L of 1 mg/mL (6.5 \( \mu \)M) the appropriate antibody (Table S2) were added per mL of AuNPs, for a final antibody concentration of 0.065 \( \mu \)M and the solution was left to shake gently at room temperature for an hour. Following this time 10 \( \mu \)L of 1 mg/mL (0.2 mM) MeO-PEG\(_{5000}\)-SH (NanoCS, product code: PG1-TH-5k) were added and the dispersion for a final concentration of 0.002 \( \mu \)M. The dispersion was left to react in the above conditions for a further hour. Particles were washed twice with PBS via centrifugation (8 000 rpm for 10 minutes). The dispersion was finally resuspended in 1/20th of the initial volume, e.g. 50 \( \mu \)L for a 1 mL initial volume. Vortexing and brief sonication (up to 3 seconds at a time) were used to disperse the particles when needed. The as made immunoprobes were stored in a 4°C fridge for up to three weeks.

**Particle characterization**

**UV-Vis**

2 \( \mu \)L of immunoprobes were diluted in 198 \( \mu \)L of PBS in a 96 well plate which was placed in a SpectraMax M5 (Molecular Designs) plate reader and measured from 400 to 800 nm at a 1 nm interval.

**DLS**

The dispersions were taken from the UV-Vis plate well as is and placed into a semi-micro (1.5 mL) plastic cuvette (Sigma, BR759115). 100 \( \mu \)L of PBS were added for a total volume of 300 \( \mu \)L in the cuvette. Each DLS (Horiba SZ-100) measurement was the result of 5 runs on automatic and was done at 25°C. The running medium was always specified as water while the particle material was gold (\( n = 0.2 \pm 3.32i \)).
Running dipsticks

Dipstick preparation

Nitrocellulose strips were cut with a laser cutter and assembled into dipstick assay strips as needed. The as made dipsticks were stained with 0.3 µL of 1 mg/mL of the target antibody (Table S2) at the test line and αRabbit Fc at the control line. All staining was done on the day of use. The dipsticks were left to dry for at least 10 minutes at RT prior to immersion in the sample.

Dipsticks for direct antigen binding experiments were done by pipetting 0.3 µL of the pure antigen on the test line instead of an antibody.

Sample preparation and running dipsticks

The target antigen (Table S1) was added to the sample and diluted via serial dilution in titration experiments. In a typical non-titration experiment 1 µL of a 10⁻² mg/mL antigen solution was added to 30 µL of the running medium and homogenized. For titration experiments 1 µL of 1 mg/mL antigen solution was added in 39 µL of the running medium. A serial dilution was done as 10 µL of the as made solution were placed in a tube containing 30 µL of the running medium, homogenised and the process was repeated up to nine times for a total of ten samples (nine with reducing antigen concentration and one control). The final 10 µL were discarded. The negative control contained 30 µL of the running medium.

2 µL (6.25% V/V) for single immunoprobes or 4 µL (11.8% V/V), total, for immunoprobe mixtures were pipetted to the as made antigen solution, homogenized and left stationary at RT for 10 – 30 minutes. After this time 15 µL of running buffer (a 1 to 1 volumetric mixture of 50% sucrose and 1% tween) were added. The dispersion was spun in a mini centrifuge (~ 1.5 krpm), vortexed and left for further 5 – 15 minutes. The dipsticks were immersed into the dispersion and left to run.

After the full sample volume had diffused through the paper, 1% tween 80 was ran through the dipstick to remove any non-specifically bound particles. The paper was left overnight at RT to dry.

Immunoprobe volume experiment for altered dipstick design

While optimizing the running process of the altered dipstick design the volume of added immunoprobes was varied while the sample volume was kept constant (SI Figure S9). In this case 1, 2, 4, 6 and 8 µL of immunoprobes (either individual or mixture) were placed in 30 µL of sample with an antigen concentration of 1×10⁻³ mg/mL.

Dipstick analysis

Image analysis, including for antigen titration curves

Dried dipsticks were affixed to white paper and scanned. The as obtained images were cropped so that only the nitrocellulose area was visible and analysed in ImageJ. The “mean gray value” of the measurement tool was used to obtain the grayscale values. The area of the rectangle used was fixed to be within the particle signal and kept constant for each image analysis. Shadows and edges were avoided as they introduced artefacts. The background grayscale value was evaluated by measuring a visibly “empty” area near the test
and control lines. For 3 location strips this was the bottom square location. For 5 location strips, each strip had a separate background measurement located just below or above the test area.

The results of antigen titrations were fit with a single Langmuir curve via an in-house written python script to obtain $K_D^{\text{Eff}}$ and $A$ values (SI Calculations section).

**LOD Analysis**

Test limit of detection was calculated from the $K_D^{\text{Eff}}$ and $A$ values obtained from the Langmuir fit. Equations are in the calculation section of the SI.

**Distribution analysis**

The scan of the full test strip was imported into ImageJ. Images were analysed in accordance with our previous work using the gel analysis tool in the software. The signal integration obtained from the analysis was converted to fractions in Microsoft Excel.

**Heatmap and pattern generation**

Grayscales obtained via ImageJ analysis were collected in excel, averaged by condition, immunoprobe and running medium and normalized as a percent of the highest value for each figure. Resulting % were separated in five groups, 0-20%, 21-40%, 41-60%, 61-80% and 81-100% where each, except the lowest category was assigned a darker shade of green. This way the heat map used in a figure is consistent with itself.

For example if we had five samples in a figure with an average grayscale value of 0.2, 25, 44, 79 and 105, they each would fall in a different category and have a darker shade of green, except the first value which would be white, as demonstrated below.

| Value (norm. %) | 0.2 (0.19%) | 25 (23.8%) | 44 (41.9%) | 79 (75.2%) | 105 (100%) |
|-----------------|-------------|------------|------------|------------|------------|
| Colour          |             |            |            |            |            |

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