A Point-of-Care Immunoassay for Ultra-Sensitive Detection of Ebolavirus

by

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Benjamin Yellen

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2020
ABSTRACT

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Abstract

Laboratory-enabled disease diagnosis is a cornerstone of patient care and relies on immunological techniques for detecting pathogens and quantifying biomarkers of clinical conditions. Immunoassay performance depends on the binding affinities of the molecules that capture analytical targets, and on the surfaces that interact with these sensing molecules and other components of biological samples. A lack of high-quality antibodies and an inability to prevent nonspecific protein absorption on test surfaces have hindered assay sensitivity and overall performance. Antibody technologies have advanced, driven by antibody-based drug development, but there remain major challenges in in vitro diagnostic (IVD) reagent generation. Protein-resistant surfaces have only recently been incorporated into immunoassay development, with promising results.

In this dissertation, we aimed to understand the surface properties required to develop a new generation of immunodiagnostics with high sensitivity and broad dynamic range. Our studies demonstrated that there are specific physicochemical properties a surface must possess to enable simple inkjet-based fabrication of antigen detection tests. We determined that POEGMA-based brushes possess the optimal balance between protein resistance and hydrophilicity, enabling noncovalent biofunctionalization and use in IVD applications. After identifying POEGMA as the ideal coating for antigen detection tests, we addressed several challenges related to antibody generation. We developed a
new antibody pair that targets non-overlapping epitopes of ebolavirus secreted glycoprotein (sGP), a truncated version of the structural glycoprotein that is actively secreted by infected cells in early stages of Ebola infection. These antibodies were generated by associating scFv phage-display technology with the transient expression of scFv candidates as Fc fusions in mammalian cells, followed by purification using a chromatography-free IsoTag-based system. We used a novel antibody-pairing strategy that leverages the D4 assay’s multiplexing capabilities and ease of fabrication to rapidly identify optimal capture and detection reagent pairs. Employing these reagents, we developed and validated an ultra-sensitive ebolavirus detection test based on the D4 assay format which outperformed the sensitivity of qRT-PCR. The exceptional sensitivity of this ebolavirus test was demonstrated in two independent nonhuman primate models of Ebola, validating the effectiveness of this new IVD test development workflow, which will enable rapid deployment of life-saving disease diagnostic efforts.
Dedication

To Ruby, Leo, and Einstein, for showing me that there is much more to life than work. To my parents for incommensurable love and support.
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1. Introduction

Immunoassays play a critical role in disease diagnosis, monitoring, and management[1] as they are the primary method for diagnosing most infectious diseases, and are essential for determining concentrations of proteins influenced by pathophysiologic events[2-3]. For most clinically relevant analytes, immunoassays have replaced chromatographic and colorimetric methods for detection. Among many variants of immunoassays, enzyme-linked immunosorbent assays (ELISA) are the foundation of immunoassays used in the clinic and in research due to their exceptional sensitivity and specificity, high throughput, and relatively low cost. ELISA involves the transduction of high specificity antibody–antigen binding into a color change generated by the interaction of an enzyme-linked conjugate with an enzymatic substrate. Color formation is used to measure the antigen concentration, and the high sensitivity of the assay allows quantitation of even very low concentrations of analytes such as proteins, vitamins, hormones, and drugs[4].

The success of ELISA is clear from its ubiquity in diverse life science fields; however, the shortcomings of ELISA are becoming more and more apparent with the growing demand for personalized point-of-care tests that rapidly measure target analytes at a patient’s home or in low-resource settings. ELISA involves assay procedures that are cumbersome and time consuming, requires a centralized laboratory with highly trained personnel and costly equipment, and involves unacceptable delays in returning test results, impeding critical decision making. Furthermore, ELISA suffers from an intrinsic
lack of multiplexing capabilities. There is an urgent need for new assay platforms to replace ELISA and allow simultaneously quantifying multiple analytes related to a pathology.

A key element of immunoassay design is the reduction of nonspecific protein adsorption (NSPA). In ELISA, reduction of NSPA is accomplished by incubating capture reagent-coated plates with blocking buffers containing albumin or casein. This blocking step increases the signal-to-noise ratio of the assay by reducing background noise caused by adventitious protein adsorption.

Due to the importance of NSPA reduction for in vitro diagnostics, much effort has been dedicated to understanding the physicochemical events related to protein adsorption, with the end-goal of creating non-fouling or low-fouling surfaces[5-7]. The rational design of non-fouling surface coatings using synthetic polymers is an important component of developing the next generation of ultra-sensitive, easy-to-use, multiplexable, and low-cost immunoassays to enable earlier disease diagnosis, improved clinical interventions, and better patient outcomes.

1.1 Protein-resistant surfaces and polymer brushes

Protein adsorption onto surfaces results from their favorable interaction with the substrate’s solvent-exposed surface. For adsorption to occur, the proteins must mainly overcome the solvation energy and penalty due osmotic pressure in hydrated surfaces. Adsorption is often a multistage process, with an initial interaction in which the protein
binds the surface, followed by alterations in protein conformation that further stabilize the immobilization[8-9]. Due to high levels of hydration (bound molecular water), highly polar groups on the substrate surface increase the free energy gradient for protein adsorption, reducing nonspecific binding. This phenomenon is ubiquitous in biology as many biological molecules have high levels of hydration to maintain their folded stability in aqueous environments. Of relevance to non-fouling surfaces, a hydrated, hydrophilic interface creates low interfacial tension between the aqueous solution and the surface, reducing the probability of interactions between the surface and proteins in the aqueous environment. In contrast, hydrophobic surfaces tend to prefer protein contacts to bound water, especially if the protein undergoes restructuring to expose hydrophobic residues that interact with the surface in an energetically favorable manner[10-11].

The most widely used polymer for conferring protein resistance to substrates is polyethylene glycol (PEG)[12-13]. PEG’s protein resistance has been attributed to its highly hydrated and randomly oriented structure, combined with its “molecular cilia” behavior in which rapid movement of the polymeric chains and high level of hydration repels proteins close to the surface. PEG surface modification can be performed using number of strategies, including “grafting to” and “grafting from” approaches. The most popular “grafting to” techniques include physisorption[14], chemisorption[15], and covalent grafting[16-17]. These polymer immobilization methods (especially covalent grafting) create strongly bound polymer chains. “Grafting to” approaches usually suffer
from low polymer density due to the diffusion-limited nature of the immobilization process\[18\]. In this approach, during immobilization, mushroom-like structures are formed by the long chains of the polymer, sterically hindering the approach of other chains in their radius and preventing their attachment. The resulting PEG surface densities are typically <4 \(\mu\)g/cm\(^2\), and have limited effectiveness in preventing nonspecific binding upon exposure to complex biological milieu\[19\].

To create more protein-resistant surfaces, methods were developed to modify surfaces using polymeric self-assembled monolayers (SAMs) which are “grafted to” the surface. An initial demonstration of improved protein resistance conferred by oligoethylene glycol-terminated alkanethiols (OEG-SH) on gold led to additional studies using SAMs\[20-21\] and a better understanding of the principles governing protein absorption on surfaces. Surface plasmon resonance (SPR) analysis on gold-grafted OEG-SH allowed identification of specific parameters that are important in non-fouling coatings. For example, self-assembling polymers containing a hydrophilic terminal group with hydrophilic internal units and a high packing density were identified as essential requirements in preventing protein adsorption\[22\].

In contrast to this “grafting to” approach, the “grafting from” strategy requires attaching polymerization initiators to the surface, from which macromolecules are subsequently grown. This approach overcomes the “grafting to” limitation of attaching pre-formed polymers to surfaces, thus enabling much higher polymer densities and
reducing surface defects. The combination of living radical polymerization (LRP)[23-24] using SAMs that created dense coatings and carry initiator groups for LRP, and the improved understanding of the molecular requirements for creating protein-resistant coatings paved the way for the rational design of new non-fouling surfaces.

Polymer brushes created by surface-initiated polymerization (SIP) emerged as the best candidate to combine the ease of formation of SAMs and polymer coatings that are both dense and thick. Polymer brushes are composed of long chains formed by combining monomeric units attached to a solid substrate; their architecture and overall properties such as grafting density, coating thickness, and hydrophilicity can be precisely controlled due to the high level of development that LRP has reached[25].

The availability of well-controlled radical polymerization techniques is largely due to the development of a diverse family of initiators[26]. Many polymerization mechanisms have been performed in SIP format, including living cationic and anionic polymerization[27-28] but the most popular methods are atom-transfer radical polymerization (ATRP) [29-30], reversible addition-fragmentation chain-transfer (RAFT) [31-32], and nitroxide mediated polymerization (NMP)[33-34]. Most of these techniques have been used to coat substrates with polymer brushes to create fouling or non-fouling surfaces[35-36].

For surfaces to be useful in clinical diagnostics, surface functionalization is required to attach reagents involved in detection of biomolecules from complex milieu.
Many strategies have been used to functionalize polymer brushes for this purpose, most involving the use of charged monomers and direct immobilization of protein without the need for coupling agents[37-38]. Some examples include the use of hydroxy-terminated poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA-OH), which is non-fouling and possess coupling agents to covalently immobilize proteins to the surface[39], and attaching biotin using N,N-disuccinimidyl carbonate (DSC) coupling to the polymer’s terminal hydroxyl group to create an anchor so that proteins conjugated to streptavidin can be immobilized onto the surface[40-41]. These strategies, which require covalent coupling of molecules to a polymer brush, can be difficult to implement due to the need for multiple processing steps and complicated chemical reactions.

1.2 POEGMA bottleneck brushes

The extensive prior work performed using PEG and SAMs laid the foundation for our group’s interest in the rational design of non-fouling surface coatings, which has expanded over the last 15 years. Previous work combined the ease of formation of SAMs with thick and robust polymer coatings to engineer a protein-resistant surface. Living radical polymerization in SIP format allowed synthesis of high-density polymer brushes with accurate control of thickness and composition. Among the different formats of LRP which include living cationic, anionic, ring-opening metathesis, free radical, and atom transfer polymerization (ATRP), ATRP was selected for synthesis of non-fouling coatings for several reasons, including (1) ATRP initiators can be easily synthesized and
immobilized onto surfaces using different methods such as plasma grafting, covalent coupling, Langmuir-Blodgett deposition, and chemical self-assembly; (2) ATRP is a living polymerization allowing control of thickness and polymer graft density; and (3) ATRP can be performed at room temperature using a number of different solvents.

Initial work from our group applied surface-initiated atom transfer radical polymerization (SI-ATRP) to grow POEGMA brushes from gold, glass, and silicon oxide surfaces. These were the first demonstrations of the non-fouling characteristics of bottleneck brushes[42-43]. Although other previous work demonstrated the use of SIP to create etch resists, coatings for corrosion control, and colloid stabilizers, there had been no reports of SIP being applied to synthesize protein- and cell-resistant polymer brushes. To grow brushes on substrates, different initiators with appropriate functional groups for SAM formation were chosen. Attachment of initiator was performed by synthesizing bifunctional molecules with the ATRP initiator at one end (bromoisobutyrate moiety) and a thiol or silane at the other end to form an alkanethiol layer on gold or a silane layer on glass (Figure 1).

A subsequent study by our group showed that protein resistance depended on brush thickness and density; this work involved varying polymerization times and the surface density of the active initiator (Figure 2). This work employed a “dummy initiator” which presented a thiol group at only one end. The results demonstrated that even low active initiator densities are sufficient to create a non-fouling surface due to the large
footprint of the brush polymer, and confirmed SI-ATRP as a robust approach to generate non-fouling surface coatings.

Figure 1: Synthesis of non-fouling bottleneck polymer brushes. (A) Surface-initiated atom transfer radical polymerization (SI-ATRP) reaction strategy used to create non-fouling bottleneck polymer brushes. (B) Thiol-terminated initiator and (C) silane-terminated initiator used to attach polymers to gold and silane oxide substrates, respectively.[43]

Figure 2: ATRP initiator density and protein resistance. (A) Polymerization strategy used to vary initiator density on substrates. (1) A bromoisobutyrate moiety was added to an alkanethiol to create the active initiator. (2) An alkanethiol without the bromoisobutyrate initiator group was used to create a “dummy” initiator to control the active initiator density on the substrate. (B) Brush thickness (Z-axis) versus polymerization time (X-axis) and active/dummy initiator ratio (Y-axis). (C) Ellipsometry measurements of silicon oxide substrates modified with the POEGMA brush. The Y-axis shows the thickness of the absorbed protein layer.[42]
Our group expanded the range of materials that support formation of POEGMA brushes with the end goal of widening the potential applications of this technology. To this end, our group developed facile methods for POEGMA brush growth and preservation of non-fouling properties on plastics[44] and high-κ dielectric materials such as TiO$_2$, ZrO$_2$, and Al$_2$O$_3$[45]. These studies further demonstrated that SI-ATRP is a robust approach for generating protein- and cell-resistant POEGMA coatings on substrates for diverse biomedical applications.

1.3 POEGMA brushes as solid phase in IVD

Increasing the sensitivity of clinical diagnostic tests enables earlier diagnosis of diseases and pre-existing conditions, and leads to early intervention, improved outcomes, and lower overall healthcare costs[46]. Improved diagnostic sensitivity has traditionally been accomplished by using signal enhancement techniques, but such methods often raise the noise of the system caused by nonspecific protein adsorption. The use of non-fouling surfaces can increase signal-to-noise while reducing the need for complex signal amplification procedures[6, 47-48].

A significant challenge involved in using non-fouling surfaces in IVD assays is enabling biofunctionalization for covalent attachment of relevant probes[49-51]. Our group sought to circumvent the need for complex chemical reactions that could compromise the non-fouling nature of the brush coating and the activity of the assay capture reagents, and reduce the throughput of device fabrication. To this end, Hucknall
et al. devised a simple method of fabricating antibody (Ab)-based microarrays which involved immobilization of Abs on POEGMA by direct noncontact printing followed by mild desiccation[6, 52]. To test the hypothesis that non-fouling surfaces could improve the sensitivity of immunoassays by reducing the adventitious adsorption of proteins, antibody arrays for IL-1β, IL-6, IL-8, TNF-α, and osteoprotegerin (OPG) were directly spotted onto 100 nm-thick brushes grown from glass, and were used in a sandwich immunoassay. Nitrocellulose was used for comparison due to its widespread use. The spotting procedure on the surface was followed by mild vacuum desiccation. This drying step removes enough water for the Abs to become entangled within the polymer and thus immobilized, while maintaining enough hydration to preserve biological activity.

The assay performance achieved using this biofunctionalization strategy was striking (Figure 3). Although the spot intensity was higher on nitrocellulose than on the POEGMA surfaces, the background signal was much lower on the POEGMA surfaces than on nitrocellulose. The result: a 100-fold lower limit of detection (LoD) for assays using the POEGMA surface. Dose-response curves in buffer and in whole serum showed similar LoDs, a promising result since complex biological samples typically yield much higher LoDs than possible using analyte-spiked buffer due to nonspecific binding. This novel approach addressed several key limitations of protein microarrays. By printing Abs directly onto dried POEGMA brushes, the susceptibility of Abs to denature upon adsorption was avoided. This also obviated the need for complex surface chemistry and
activation/inactivation steps. When the POEGMA brushes were hydrated, the non-fouling properties of the surface prevented nonspecific protein adsorption. Separate blocking steps after capture reagent immobilization became unnecessary due to the dramatically reduced NSPA.

**Figure 3**: Protein microarrays on POEGMA brushes. (A) Image of IL-6 microarray assay using POEGMA-modified surfaces in serum, showing the relationship between IL-6 concentration and fluorescence intensity. (B) Dose-response curve of osteoprotegerin (OPG) in serum and in buffer, showing comparable limits of detection independent of sample complexity. (C) Dose-response curves for IL-6 in serum on POEGMA brushes and on nitrocellulose membrane, showing a lower limit of detection and greater dynamic range using the non-fouling POEGMA surface.[52]

### 1.4 The D4 point-of-care assay

Following these demonstrations of simple fabrication of fluorescent antibody microarrays with POEGMA-modified surfaces, our group sought to apply the POEGMA brushes and simple biofunctionalization strategy to point-of-care device platforms that address the urgent need for ELISA-like sensitivity together with the ease-of-use of devices based on lateral flow assay (LFA) technology.

Lateral flow assays require minimal user intervention and provide qualitative or semi-quantitative readouts within 30 min with little user training. Their ease of use,
simple design, low fabrication cost, and reliability have made LFA technology commonly adopted for field use, with diverse applications from infectious disease diagnostics to environmental monitoring to food testing. LFA use has become so ubiquitous that it is not uncommon to see LFAs used for infectious disease diagnosis in emergency rooms in the US and in clinical laboratories in developing countries[53-54]. Although LFAs have had a positive impact and are key in providing life-saving access to diagnostics, especially to underprivileged populations, these tests possess an intrinsic lack of analytical sensitivity, which limits their use to detecting only targets present at high concentrations, excluding many diseases and preventing early diagnoses. Several clinical conditions require accurate quantitative assessment of the target analyte which LFAs have thus far been unable to deliver reliably, even with the use of LFA readers[55-56].

To develop a test that rivals the portability of LFAs and the sensitivity of ELISA, our group reimagined the traditional immunoassay to create the D4 assay point-of-care platform. To this end, Joh et al. fabricated protein microarrays in which capture Ab (cAb) was printed onto a POEGMA-coated substrate to generate “stable” capture spots, and fluorescently-labelled detection Ab (FL-dAb) was mixed with an excess of soluble PEG or trehalose (to re-solubilize the FL-dAb upon sample exposure) and was printed onto the POEGMA brush to create “soluble” detection spots in close proximity to the cAb[57]. With this new architecture, all reagents required in the assay were built into the test
device, obviating the need for wash and incubation steps that are required in ELISAs and similar protein microarrays.

The D4 assay is so named due to the chain of events that drive the assay to completion (Figure 4A-D). Once the sample is dispensed onto the surface of the chip, the “soluble” detection spots dissolve upon contact with blood or other sample. The FL-dAb diffuses through the surface of the chip and binds to the cAb-bound analyte. Following a rinse step, the assay fluorescence is detected by using a tabletop or handheld fluorescence scanner.

**Figure 4:** D4 immunoassay on POEGMA brushes. (A) cAb and dAb are printed directly onto the POEGMA brush. (B) A drop of blood or serum is dispensed directly onto the chip. (C) The dAb dissolves and binds antigen in the sample. (D) Ab-Ag complexes diffuse and bind cAb spots. (E) D4 chip fabrication. A POEGMA brush is grown on glass chips using SI-ATRP. The cAb and dAb are inkjet-printed onto the surface. After desiccation, the D4 chips are ready for use. [57]
Figure 4E depicts the fabrication of the D4 assay, including the growth of the POEGMA brush on a glass slide, followed by inkjet printing of cAb and FL-dAb directly on the surface of the chip. Following a mild desiccation step, the assay is ready for use. Fabrication is simple and consumes small amounts of reagents (pg–ng of antibody), translating into production at scale and low cost per fabricated test, key elements in delivering a platform that can rival current POCTs.

The D4 assay on the non-fouling POEMGA brush achieved femtomolar sensitivity with 4-log dynamic range (DR) in IL-6 detection in whole chicken blood and fetal bovine serum (Figure 5).

Figure 5. D4 assay of IL-6 in whole serum and blood. (A) D4 assay image data. Spots of Cy5-dAb against human IL-6 are printed around spots of anti–IL-6 cAb. Spots of PBS and anti-dAb Abs printed nearby serve as negative and positive controls. All spots are outlined by a dashed white line to aid visualization. Images show arrays exposed to whole chicken blood alone (left) and blood (right) spiked with IL-6. (B) Spatial intensity plots of fluorescence from individual cAb spots at various concentrations of IL-6 spiked in whole chicken blood. (C and D) Dose–
response curves of IL-6 in (C) calf serum and (D) chicken blood. Each data point represents mean ± SD from three D4 array runs. LODs in serum and blood were 6.3 and 10.9 pg/mL, respectively. [57]

A key element of the D4 assay as a novel protein microarray format is its intrinsic multiplexing capabilities, since the capture spots for each target analyte can be printed spatially separated, and a mix of FL-dAbs are printed in a circular pattern around the cAbs. This configuration allows the use of one fluorescent probe for all dAbs. Simultaneously detecting multiple biomarkers has widespread clinical applications, as detection of only a single analyte can yield poor clinical sensitivity and specificity. To demonstrate a multiplex D4 assay, Joh et al. simultaneously detected IL-6 and TNF-α, with comparable performance to single-analyte versions of the test (Figure 6). The D4 assay showed equivalent dose-response behavior and performance in whole blood with IL-6 or TNF-α alone and with both simultaneously and showed minimal cross-reactivity.
Figure 6: Multiplexed assays against cytokines IL-6 and TNFα in whole blood. Fluorescent detection reagents against both analytes are coprinted as outer spots. Spots of cAb against IL-6 and TNFα are printed in the center of the array. (A) D4 image after incubation with whole chicken blood alone (without analyte). Spots for cAb and PBS are indicated by white dotted lines. (B) Dose–response curves after exposure to whole chicken blood spiked with a mixture of IL-6 and TNFα at different concentrations. (C and D) Dose–response curves after exposure to different concentrations of (C) IL-6 only or (D) TNFα only. Insets for panels B–D show D4 image data. Each data point represents mean ± SD from three D4 assay runs. [57]

Following development and characterization of the performance of the D4 assay, our group ran a pilot clinical study to ascertain the feasibility of using the D4 assay as a POCT platform. To this end, the D4 assay was used to quantitatively determine leptin levels in patient serum, and those results were compared with standard laboratory techniques (Figure 7). D4 assay output fluorescence showed a strong correlation with analyte concentration determined by ELISA. The D4 chips were also compatible with a low-cost phone-based fluorescence detector, although there was a loss in sensitivity when compared to a tabletop scanner.
Figure 7: Mobile phone-based imaging of D4 arrays. (A and B) Image data of D4 microspots and dose–response curves in calf serum spiked with leptin (analyte) acquired using (A) a benchtop scanner and (B) a mobile phone-based fluorescence microscope. A five-parameter logistic fit is shown as the red curve. (B, inset) Illustration of mobile phone attachment for D4 array imaging and quantification. (C) Testing of obese patient serum with D4 assay using a scanner (red trace) versus a mobile phone microscope (blue trace), and comparison with ELISA results (dashed black trace). Normalized D4 data (scanner, phone) is plotted on the left axis; normalized ELISA data is plotted on the right axis. [57]

1.5 Conclusions

The limitations of traditional IVD tests, which lack ease-of-use and portability for point-of-care applications, or which lack sensitivity and the ability to accurately quantify analytes motivated our group to employ non-fouling POEMGA surfaces to generate IVD tests that are easy to fabricate. As all reagents are already inkjet-printed on the surface of the chip, the assay is easy to use—the user only needs to add the sample and the assay
goes to completion. The assay yields femtomolar LoDs and large DRs as the POEGMA coating blocks nonspecific binding of proteins to increase the signal-to-noise and improve the performance of the assay.

Understanding that non-fouling coatings could be key to the future of immunoassays, we extended our work using POEGMA brushes and the D4 assay to further comprehend the properties that drive non-covalent biofunctionalization and simple fabrication of these devices. Followed by additional attempts to address large challenges in the field of diagnostics such as rapid paired reagent generation for sandwich immunoassays and ultra-sensitive infectious disease detection, described below.
2. Engineering zwitterionic brush surface properties for protein microarrays

Many approaches have been used to generate protein-resistant coatings, including covalent conjugation of the “stealth” polymer, poly(ethylene glycol)[58-59]; adsorption of PEG-based surfactants such as Pluronics[60-61] onto surfaces; electrostatic adsorption of PEG-functionalized polymers[62-63]; and SAMs on gold that present terminal oligoethylene glycol moieties[21, 64]. The only initial approach that eliminated NSPA was the use of OEG-SAMs restricted to gold and silver substrates. This first generation of protein-resistant coating was followed by the use POEGMA brushes synthesized using SI polymerization, which our group pioneered. Early work demonstrated that POEGMA coatings synthesized by SI-ATRP with thickness ≥10 nm exhibit exceptional protein and cell resistance, even when exposed to complex samples such as whole blood, serum, and plasma[65]. This work was followed by demonstration of the utility of POEGMA coatings in IVD tests, initially by creating a protein microarray using noncovalent biofunctionalization of the brushes. Although the resulting assay showed exceptional sensitivity, this assay format required multiple incubation and washing steps, and thus unsuitable for field use. Next, our lab developed the D4 assay, in which all reagents are inkjet-printed on-chip to integrate all components required for a sandwich immunoassay in a POCT format, addressing the need for a test platform that reduced user intervention.
After development of the D4 assay on POEGMA brushes, our group sought to further understand the physicochemical requirements of brush coatings that enable easy fabrication of IVD tests. To this end, we explored the use of zwitterionic brushes (ZIB) for D4 fabrication, followed by engineering the surface properties of hybrid cationic-zwitterionic brushes to enable inkjet printing of capture and detection reagents, thus expanding the potential coatings that can be used for D4 assay fabrication. We were also interested in alternatives to brushes with PEG side chains, as these constructs present a few important limitations in a bioanalytical setting: First, PEG auto-oxidizes into reactive groups when exposed to oxygen and transition metal ions present in biological samples, potentially limiting the assay shelf-life[66]. Second, the ubiquitous use of PEG in consumer and food products has led to widespread prevalence of circulating anti-PEG antibodies[67-70]; this Ab formation also occurs on POEGMA coatings with side chains longer than three ethylene glycol repeats[71]. These anti-PEG Abs that directly bind to POEGMA brushes can interfere with immunoassays.

Our choice of ZIB was motivated by reports of their exquisite resistance to the nonspecific adsorption of proteins and adherence of cells[72]. Although ZIB-coated surfaces have been employed in immunodiagnostics by using covalent conjugation of capture reagents to surfaces[73], this strategy is limited by the need to activate the surface for conjugation followed by deactivation of reactive moieties prior to use. Chemical coupling of capture reagents also makes multiplexing more difficult because of the need
for sequential coupling of different capture moieties to spatially-defined areas of the substrate. Although ZIBs are promising, their intrinsic high wettability and extremely low contact angles[74] could pose challenges, especially considering the non-contact dispensing approach to functionalize the surfaces. For example, the commonly used “non-fouling” ZIB poly(sulfobetaine)methacrylate (PSBMA) is far too hydrophilic to allow noncovalent functionalization of antibodies by inkjet printing. To overcome this limitation, we explored alternative surfaces that enable D4 assay fabrication, which culminated in the development and characterization of a series of new hybrid zwitterionic-cationic polymer brush coatings with tunable surface wettability suitable for inkjet printing of antibodies. We showed that a point-of-care microarray immunoassay fabricated on these hybrid brushes has a similar analytical sensitivity and dynamic range as assays fabricated on POEGMA brushes. We also demonstrated that the mixed ZIB brushes do not bind the anti-PEG antibodies that are ubiquitous in human blood, unlike POEGMA brushes or other PEG-functionalized surface coatings.

2.1 Results and Discussion

Initially, we employed inkjet printing of capture (Ab<sub>c</sub>) and detection antibodies (Ab<sub>d</sub>) on zwitterionic (ZI)-polymer coated surfaces to create D4 assay chips, in a similar fashion to the fabrication strategy employed on POEGMA brush surfaces. To this end, we performed ATRP using sulfobetaine methacrylate (SBMA) on glass slides with a covalently immobilized ATRP initiator (APTES-BiB) to create a PSBMA brush. We
selected SBMA rather than carboxybetaine methacrylate (CBMA) as monomer to create a nonfouling ZIB due to SBMA’s commercial availability and low cost. A summary of the synthesis route of PSBMA brushes on glass is shown in Figure 8A, and employs SBMA as the monomer, a mixture of Cu(I) and Cu(II) transition metal catalysts, and HMTETA as the ATRP ligand in a methanol/water cosolvent. With this approach we were able to synthesize PSBMA films with tunable thickness by varying the polymerization time. Figure 8B shows the polymer brush thickness on SiO₂ as a function of polymerization time, measured using reflective mode ellipsometry in air. The thickness of PSBMA increases monotonically as a function of polymerization time, reaching ~40 nm in just under 16 h. Although effective in producing films with tunable thicknesses, the “classical” ATRP methodology used here has undesired features, including the need for a high concentration of transition metal catalyst, and an oxygen-free atmosphere to perform the reaction[29, 75].
Figure 8: Polymer brushes grown from silicon oxide and glass substrates. (A) Stepwise strategy of PSBMA brush synthesis on thermally-deposited SiO$_2$ on Si, and on glass slides. Surface functionalization with APTES allows installation of the ATRP initiator BiB ($\alpha$-bromoisobutyryl bromide). PSBMA brushes are then “grafted from” the surface by ATRP. (B) PSBMA brush growth on SiO$_2$ was investigated by reflective mode ellipsometry. Each data point is the average of three independent measurements. Abbreviations: EtOH = ethanol, MeOH = methanol, DCM = dichloromethane, TEA = triethylamine, Cu(I)Br = copper I bromide; Cu(II)Br = copper II bromide; HMTETA = 1,1,4,7,10,10-hexamethyltriethylenetetramine, SA = sodium ascorbate, SBMA = sulfobetaine methacrylate, CBMA = carboxibetaine methacrylate.

An alternative route for this polymerization is the use of activators regenerated by electron transfer (ARGET) ATRP, which can be carried out in an aqueous environment, uses a low concentration of transition metals, and is oxygen tolerant, resulting in a more environmentally friendly process with less need for solution deoxygenation[77]. We
attempted to perform ARGET-ATRP of SBMA brushes, but observed early termination, resulting in polymer brushes with thicknesses of 3–7 nm. We abandoned this approach because our previous work on POEGMA brushes had shown that a polymer film thickness >10 nm is required for protein resistance[42].

Following PSBMA brush synthesis, we characterized the polymer-coated glass substrate. We performed X-ray photoelectron spectroscopy (XPS) to analyze the molecular composition of the initiator- and PSBMA-functionalized substrate. Figure 9A shows the survey scan spectrum of SiO$_2$ after functionalization with APTES-BiB prior to ATRP. The spectrum exhibits characteristic peaks associated with the SiO$_2$ substrate, and a small nitrogen peak at ~399 eV that can be attributed to the nitrogen moiety in the surface-immobilized APTES. Figure 9B-C show survey spectra of 11 nm- and 33 nm-thick PSBMA brushes; insets depict high-resolution scans for the N$_{ls}$ peak. As reported elsewhere, the bromide peak was not visible in the survey scan spectra[45]. Survey scans of the PSBMA brush-coated surfaces show large nitrogen and sulfur peaks as expected from the polymer composition. The 11 nm-thick PSBMA coating has small Si peaks which are absent in the survey scan spectrum of the 33 nm PSBMA coating, consistent with the ~10 nm sampling depth of XPS[78]. High-resolution scans of 11 nm-thick PSBMA coatings show a dominant quaternary amine peak (402.0 eV) originating from the polymer coating and a small tertiary amine peak (398.6 eV) associated with the underlying APTES-BiB
This latter peak is not detected with 33 nm brushes, corroborating the results obtained from the survey scan spectra.

**Figure 9:** XPS characterization of PSBMA brush-coated substrate. (A) Survey scan spectrum of APTES-BiB (initiator) covalently attached to an SiO\(_2\) surface. (B) Survey scan spectrum of 11 nm-thick PSBMA coating shows reduction in the intensity of O1s, Si2s, and Si2p peaks, an increase in the intensity of the N1s peak compared to the APTES-BiB modified glass surface, and the appearance of S2s and S2p peaks, consistent with formation of a PSBMA overlayer. Inset: High resolution N1s spectrum of PSBMA with peak fitting, showing a large component peak corresponding to quaternary amines, consistent with the chemical structure of PSBMA, and a smaller component peak corresponding to tertiary amine groups from the APTES-BiB underlayer. (C) Survey scan spectrum of 33 nm-thick PSBMA coating on glass shows characteristic S2s and S2p from the PSBMA overlayer and the absence of silicon peaks, consistent with the PSMA overlayer being greater than the XPS sampling depth. Inset: High resolution N1s spectrum with peak fitting following ATRP, showing the prevalence of quaternary amines, consistent with formation of a PSBMA overlayer.

Abbreviations: N1s peak N = tertiary amine, N+ = quaternary amine. [76]

We next investigated the protein resistance of each PSBMA-modified surface. We incubated bare glass slides, glass functionalized with APTES-BiB, and glass with ~40 nm thick POEGMA and PSBMA polymer brushes grafted from the surface in a solution of 10 mg/mL FITC-BSA in PBS for 24 h at room temperature. Following a wash with PBS and Tween-20 to remove unbound protein, the surfaces were imaged using a fluorescent slide
scanner to measure BSA adsorption. Figure 10A shows low fluorescence intensity on POEGMA and PSBMA polymer brush surfaces, and much higher intensity on APTES- and APTES-BiB-functionalized substrates. These results, quantified in Figure 10B, showed that protein adsorption was significantly greater on the APTES- and APTES-BiB-functionalized surfaces than on the PSBMA and POEGMA brush-coated surfaces (p<0.001). There was no statistically significant difference between BSA adsorption on bare glass, POEGMA, and PSBMA. POEGMA-coated surfaces showed a slightly lower fluorescence than PSBMA, but this effect was not statistically significant, consistent with a previous study[79].

![Figure 10: Protein adsorption and anti-PEG Ab binding on POEGMA and PSBMA brushes. (A) Fluorescence images and (B) quantification of adsorption of FITC-BSA (10 mg/mL in PBS) on glass, APTES and APTES-BiB functionalized glass,]
and POEGMA and PSBMA brushes. There is a statistically significant difference between group mean FITC-BSA adsorption as determined by one-way ANOVA followed by Tukey’s post-hoc test (p<0.001). Means with lines between bars and marked with a * are significantly different. (C) Fluorescence images and (D) quantification of anti-PEG Ab binding on POEGMA and PSBMA. There is a statistically significant difference between group means as determined by one-way ANOVA followed by Tukey’s post-hoc test (p<0.001). [76]

Next, we tested the reactivity of PSBMA brushes towards anti-PEG Abs. We incubated PSBMA- and POEGMA-coated glass slides with rabbit anti-PEG polyclonal Abs for 1 h, rinsed the surfaces with 0.1% Tween-20 (v/v) in PBS, then incubated with an Alexa Fluor 647-labeled anti-rabbit secondary Ab in PBS for 1 h. We incubated a POEGMA-coated glass slide with only a fluorescently-labelled anti-rabbit Ab as a control for nonspecific binding of detection antibody (labeled POEGMA(c) in Figure 10D). Following a final wash with 0.1% Tween-20 (v/v) in PBS, the substrates were spun-dry and imaged using a slide scanner. Figure 10C shows the level of fluorescence on the POEGMA and PSBMA surfaces. The PSBMA coating on glass showed a low fluorescence intensity, indicating low reactivity towards anti-PEG Abs (Figure 10C). The POEGMA-coated glass slide showed a high intensity (which saturated the detector) due to the binding of PEG side chains by the anti-PEG primary Ab. Quantitation of fluorescence intensity showed that binding of anti-PEG Ab was significantly greater on POEGMA-coated glass than on PSBMA-coated glass and on POEGMA(c), the negative control (p<0.0001) (Figure 10D). Binding of anti-PEG Ab on PSBMA-coated glass and on the negative control, POEGMA(c), were not significantly different.
After observing low protein adsorption and low anti-PEG binding on PSBMA-coated glass, we fabricated D4 assays for detection of IL-6 on POEGMA- and PSBMA-coated glass slides. We spotted a cAb for IL-6 in the central region of the chip and Alexa Fluor 647-labeled cAb in concentric patterns around the cAb capture spots. We ran the D4 assay by adding calf serum spiked with IL-6 to the chip surface. Figure 11A shows a D4 chip on POEGMA with the inkjet-printed cAb, PBS, and dAb spots spatially separated on the chip surface. The image shows a low background and a high signal-to-noise after incubation with calf serum spiked with 3.9 ng/mL IL-6. The corresponding dose-response curve for detection of IL-6 (Figure 11B) showed an LoD of 26 pg/mL and DR of 3.4, demonstrating the exceptional performance of this assay fabricated on a POEGMA-coated surface.

In contrast to the POEGMA-coated surface, the PSBMA brush surface yielded D4 assay capture spots that were barely visible after completion of the IL-6 assay, and detection spots with much lower intensity and larger diameter than those on the POEGMA surface (Figure 11C). The corresponding dose-response curve for IL-6 (Figure 11D) shows that inkjet printing of the cAb and dAb on the PSBMA-coated chips does not yield a usable assay. Based on the spot morphology (Figure 12), this failure of the D4 assay on the PSBMA-coated surface is liked due to its hydrophilicity and the consequent lack of stable cAb entrapment in the polymer brush.
To test this hypothesis, we measured the water contact angle in air for POEGMA- and PSBMA-coated glass slides using the sessile drop technique (Figure 11E). The contact angle of the PSBMA-coated surface is ~8° and that of the POEGMA-coated surface is ~44° (Figure 11F), confirming that the PSBMA surface is far more hydrophilic than the POEGMA surface. The extremely low contact angle of the PSBMA-coated surface leads to spot sizes of ~300 μm for the cAb and dAb, significantly larger than the ~180 μm spots on the POEGMA surface (ANOVA followed by Sidak’s post-hoc test, p<0.0001). These results showed that inkjet printing of cAb and dAb on the extremely hydrophilic PSBMA polymer coating was not feasible.

Figure 11: D4 assay on POEGMA and PSBMA brushes. (A-D) Fluorescence images and dose-response curves of D4 microarrays against IL-6 fabricated on POEGMA (A-B) and PSBMA (C-D) brushes. Alexa Fluor 647-conjugated detection antibodies are spotted dose-response curves (B, D) of D4 microarrays against IL-6 fabricated on POEGMA (A-B) and PSBMA (C-D) brushes. Alexa Fluor 647-conjugated
detection antibodies are spotted around unlabelled capture antibody and phosphate buffered saline (PBS) spots (negative control). Capture and PBS spots are outlined by dashed circles. A 5-parameter logistic fit for the dose-response curve in panel B is shown by the blue curve. Each data point represents the average and standard error of four independent D4 assays. (E-F) Optical images and quantification of contact angles on POEGMA and PSBMA polymer brushes. There were statistically significant differences between group means as determined by Student t-test (p<0.001). Means with lines between bars and marked with a * are significantly different. [76]

Figure 12: Capture and detection spots on POEGMA and PSBMA substrates imaged before and after sample incubation. (A) Optical and fluorescence images obtained before and after sample incubation depicting the difference in capture and detection spot morphology for D4 assays on POEGMA and PSBMA substrates. Both substrate were inkjet-printed in the same print run, with drops of 350 pL for capture and 300 pL for detection spots. (B) Spot size evaluated for D4 assays fabricated on POEGMA and PSBMA spot sample incubation. Independent measurements were performed in 5 different chips. There were statistically significant differences between group means as determined by two-way ANOVA (p<0.001) followed by Sidak’s post-hoc test. Means with lines between bars and marked with a * are significantly different. [76]
To address the problem of inkjet printing on the PSBMA surface—and potentially on other highly hydrophilic ZI polymer coatings—we developed a new route to synthesize tunable “hybrid” polymer brushes that present a protein-resistant zwitterionic side chain on a polymer backbone. This approach involved the synthesis of a hybrid polymer brush that contains ZI and amine groups, which we hypothesized would allow immobilization of proteins via inkjet printing due to electrostatic and hydrogen bonding with antibodies, but would also present a sufficiently high concentration of ZI side chains to provide protein resistance in the background regions not printed with assay reagents.

Figure 13A depicts the synthesis strategy used to implement this approach, where poly-2-(dimethylamino)ethyl methacrylate (PDMAEMA) brushes with amine groups in the side chains are grafted from the surface by ARGET-ATRP, and are reacted with iodoacetic acid under different conditions of pH and temperature to generate PCBMA-PDMAEMA hybrid brushes with different fractions of amine-terminated and ZI CBMA side chains. We hypothesized that higher pH and temperature in the derivatization reaction would yield higher degrees of conversion of the amine into zwitterionic CBMA groups, so that temperature and pH could be used to tune the degree of conversion and thereby tune the protein resistance, drop contact angle, and overall properties that enable printability and non-covalent biofunctionalization of the substrate.

To explore this approach, we systematically varied the polymerization time of PDMAEMA on APTES-BiB-functionalized glass slides and SiO₂ chips, thereby obtaining
PDMAEMA brushes of different thicknesses, measured using ellipsometry (Figure 13B). After PDMAEMA brushes were synthesized, we sought to control the extent of conversion of PDMAEMA to PCBMA by derivatizing the PDMAEMA-coated surfaces with 20 mM iodoacetic acid solution at a pH range of pH 5-8 and at 45°C or 75°C for 24 h. Following derivatization, we again measured the polymer brush thickness using ellipsometry and compared the thicknesses before and after conversion (Figure 13C). The brush thicknesses were significantly greater following derivatization. The cause of the increase in thickness was unclear, but we speculated that the increased thickness was due to a combination of factors: (1) the mass added to the side chains by derivatization of the amine group; (2) the increase in side chain charge, which could increase water retention—even in the macroscopically dry state that ellipsometry is performed in; and (3) a change in the polymer brush conformation to a more elongated state.
Figure 13: PDMAEMA polymer brushes grown from silicon oxide and glass and their conversion into PCBMA-PDMAEMA hybrid brushes. (A) Stepwise strategy of PCBMA-PDMAEMA hybrid brush synthesis on thermally-deposited SiO$_2$ on Si and glass slides. Oxide surface functionalization with APTES allows installation of the ATRP initiator BiB (α-bromoisobutyryl bromide). PDMAEMA brushes are then “grafted from” the surface by ATRP and later converted into PCBMA. (B) PDMAEMA brush growth on SiO$_2$ was investigated by reflective mode ellipsometry. (C) PDMAEMA brush thickness before and after conversion into PCBMA-PDMAEMA under different combinations of pH and temperatures of 45°C and 75°C. There were statistically significant differences between group means as determined by two-way ANOVA followed by Sidak’s post-hoc test (p<0.001). On a pairwise basis, the change in mean thickness before and after derivatization was statistically significant for all cases. (D) Derivatization reaction of PDMAEMA brush on glass with iodoacetic acid,
showing positively- and negatively-charged groups that form the zwitterionic chemical motif. [76]

Next, we performed XPS on ~40 nm thick PDMAEMA and mixed PCBMA-PDMAEMA brushes. Figure 14 shows the survey scan spectrum of the PDMAEMA brush, showing characteristic peaks for C1s, N1s and O1s, consistent with the polymer composition. The inset in Figure 14A shows a high-resolution N1s spectrum of the PDMAEMA brush; peak fitting showed that it could be fit with a single component that can be assigned to tertiary amines (398.6 eV), consistent with the chemical structure of PDMAEMA. Figure 14B-E show high-resolution N1s spectra of PDMAEMA derivatized with iodoacetic acid at different combinations of temperature and pH. The peak fits of the high-resolution scans show that, depending upon the specific derivatization conditions, a significant fraction of the tertiary amines (398.6 eV) are converted into quaternary amines (402.0 eV), a signature of PCBMA.

Table 1 shows the quantified XPS data, revealing that at both 45°C and 75°C the yield of the derivatization reaction shows a maximum at pH 7, leading to conversion of up to two-thirds of the tertiary amines into quaternary amines that are indicative of PCBMA.
Figure 14: XPS spectra of PDMAEMA and PCBMA-PDMAEMA after derivatization under different conditions. (A) Survey scan spectrum of PDMAEMA. Inset: High resolution N1s spectrum with peak fitting following SI-ATRP, showing the that the sole nitrogen-containing moiety is a tertiary amine, consistent with the structure of PDMAEMA. (B-E) High resolution N1s spectra with peak fitting following derivatization under different reaction conditions: (B) pH 5 at 45°C, (C) pH 5 at 75°C, (D) pH 7 at 45°C, and (E) pH 7 at 75°C, showing the appearance of a second component that can be assigned to a quaternary amine, consistent with the incorporation of a carboxylic acid with a carbon spacer moiety. Survey spectra of PDMAEMA brushes derivatized to PCBMA. [76]

Table 1: Atomic concentrations of carbon, nitrogen, oxygen, and sulfur measured using XPS survey scans. Tertiary and quaternary amine concentrations measured using high-resolution N 1s scans. Values calculated by peak deconvolution, setting C 1s peak to 284.5 eV, tertiary amine peak to ~398.6 eV, and quaternary amine
peak to \(\sim 402\) eV. Abbreviations: \(N\) = tertiary amine, \(N^+\) = quaternary amine, High Res = high resolution [76]

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<td>26.6</td>
<td>0.0</td>
<td>100</td>
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| 45°C | PCBMA pH5 | 71.8 | 6.4 | 21.8 | 0.0 | 44.2 | 55.8 |
|      | PCBMA pH6 | 70.8 | 6.1 | 23.1 | 0.0 | 61.7 | 38.3 |
|      | PCBMA pH7 | 71.0 | 5.9 | 23.1 | 0.0 | 67.0 | 33.0 |
|      | PCBMA pH8 | 70.5 | 5.9 | 23.6 | 0.0 | 62.4 | 37.6 |
| 75°C | PCBMA pH5 | 70.8 | 6.2 | 23.0 | 0.0 | 57.5 | 42.5 |
|      | PCBMA pH6 | 71.0 | 6.0 | 23.0 | 0.0 | 62.5 | 37.5 |
|      | PCBMA pH7 | 71.0 | 5.9 | 23.1 | 0.0 | 70.9 | 29.1 |
|      | PCBMA pH8 | 75.5 | 5.8 | 18.7 | 0.0 | 60.5 | 39.5 |

*The indicated values correspond to the expected stoichiometric percentages of each element.

Following XPS analysis, we measured protein adsorption, water contact angle, and anti-PEG Ab binding on PDMAEMA and ZI hybrid surfaces. Figure 15 shows fluorescence images and quantitation of FITC-BSA adsorption on these surfaces. PDMAEMA showed a high level of protein adsorption, which can be attributed to electrostatic binding of proteins to its highly charged surface. Iodoacetic acid derivatization at pH 5, 45°C caused significantly greater in protein absorption than on PDMAEMA coatings, which was unexpected as the insertion of zwitterionic motifs should increase protein resistance. However, as the temperature and pH of the derivatization reaction increases, the surfaces become more protein resistant, a trend that is clearly seen in Figure 15B and is consistent with the increasing conversion of the amine group to zwitterions (Figure 14 and Table 1). The derivatized surfaces labeled A-F in
Figure 15B have protein resistance levels that are comparable to those of PSBMA but somewhat higher than those of POEGMA.

![Figure 15](image)

**Figure 15.** Protein resistance of PDMAEMA and PCBMA-PDMAEMA brushes. (A) Fluorescence images and (B) quantification of protein resistance assays performed using FITC-BSA (10 mg/mL in PBS) on PDMAEMA before and after derivatization with iodoacetic acid under different combinations of pH and temperature. There was a statistically significant difference between group means as determined by one-way ANOVA followed by Tukey’s post-hoc test (p<0.001). Means sharing the same letters are significantly different. [76]

We next measured the sessile water contact angle on these surfaces. Figure 16A shows photographs of water drops on different surfaces, and Figure 16B shows the water contact angles on all polymer-coated substrates. The results clearly demonstrated that a higher degree of surface derivatization yields more hydrophilic surfaces, as there was a near monotonic reduction in both protein adsorption and water drop contact angle as the fraction of zwitterionic groups increased. XPS analysis showed that pH 8 at 75°C resulted
in lower derivatization ratios than pH 7, and a larger water drop contact angle was observed at pH 8; nonspecific protein binding on the substrate converted at pH 8 and 75°C was lower than at pH 7 and 75°C, although this difference was not statistically significant. These results demonstrate that there is a direct dependency between the degree of zwitterionic groups on the substrate and surface hydrophilicity, and a somewhat weaker trend with respect to protein resistance. The exception to this trend is POEGMA, which has the lowest protein adsorption but has a significantly higher water contact angle (~44°). Although hydrophilicity is important for protein resistance, it is not its sole determinant[80-85].

Figure 16. Water contact angles of PDMAEMA and PCBMA-PDMAEMA brushes. (A) Optical images and (B) sessile drop contact angles measurements on PDMAEMA brushes before and after derivatization with iodoacetic acid under different conditions of pH and temperature. There were statistically significant differences between group means as determined by one-way ANOVA followed by
Tukey’s post-hoc test (p<0.001). Means sharing the same letters are significantly different. [76]

Next, we examined the binding of anti-PEG Ab to these surfaces. As the derivatized polymer brushes do not present ethylene glycol chains in their structure, the binding of anti-PEG (and secondary Ab) can be attributed to nonspecific adsorption rather than to molecular recognition, as is the case with POEGMA coatings. Fluorescence intensity decreased with an increase in pH and temperature at which the derivatization reaction was performed (Figure 17A). The lowest nonspecific adsorption of anti-PEG observed on iodoacetic acid-derivatized PDMAEMA brushes occurred at either a high pH (pH 8) or a high temperature (75°C). Anti-PEG Ab adsorption on these derivatized surfaces was statistically indistinguishable from that on the PSBMA brush (Figure 17B).

![Figure 17: Anti-PEG Ab binding to PDMAEMA and PCBMA-PDMAEMA brushes. (A) Fluorescence images and (B) quantification of anti-PEG Ab resistance](image-url)
assays performed with polyclonal anti-PEG Abs and Alexa Fluor 647-labeled secondary Abs on PDMAEMA brushes before and after derivatization with iodoacetic acid at different combinations of pH and temperature. There were statistically significant differences between group means as determined by one-way ANOVA (p<0.001) followed by Tukey’s post-hoc test. Means sharing the same letters are significantly different. [76]

Finally, we correlated surface properties with D4 assay performance fabricated on these substrates. The immunoassay fabrication on the PCBMA-PDMAEMA brushes followed the same methodology as described in Figure 4. Dose-response curves were obtained for each substrate by incubating 80 μL of analyte-spiked IL-6 calf serum for 1 h, followed by a rinse in 0.1% Tween-20 (v/v) in PBS, and imaging using a fluorescence slide scanner. Figure 18A shows the results of a D4 assay on PDMAEMA brushes, with fluorescence images in each panel depicting the raw readout of each assay. The high level of nonspecific adsorption, visible by the red fluorescence throughout the chip surface, drowns out the signal from the printed cAb spots, making it impossible to obtain a dose-response curve.
Figure 18. D4 assay dose-response curves. (A-F) Dose-response curves of D4 assays fabricated on PDMAEMA and PCBMA-PDMAEMA brushes synthesized under different conditions of pH and temperature. Insets: fluorescence images with detection and capture reagents inkjet-printed on the brush-coated surfaces. Each point in the dose-response curve represents the average and standard error of three independently conducted D4 assays. A 5-parameter logistic fit is shown by the blue curve. [76]

Figure 18B shows the dose-response curve of a PDMAEMA substrate derivatized at 45°C and pH 5. The background fluorescence caused by nonspecific binding was significantly lower than for the native PDMAEMA chip (Figure 18A), and the capture spots were clearly visible against the background, enabling a dose response-curve to be generated, albeit with a high baseline. When the pH was held constant at 5 but the temperature of derivatization was increased to 75°C, the surface became protein-resistant to a degree that allowed a dose-response curve to be determined (Figure 18C). The background signal (seen on the inset) was low, and the capture spots could be clearly
resolved. Figure 19D-E shows the dose-response behavior obtained with other conditions, and Figure 19 depicts all dose-response curves generated in this work. Table 2 presents the figures of merit (FOM)—limit of blank (LoB), limit of detection (LoD), and dynamic range (DR)—calculated by averaging four independently determined dose-response curves, and correlates these FOMs with water contact angle (WTCA) and surface protein absorption.
Figure 19: D4 assay dose-response curves. (A-K) Dose-response curves of D4 assays fabricated on POEGMA, PSBMA, PDMAEMA, and PCBMA-PDMAEMA brushes synthesized under different conditions of pH and temperature. Each point in the dose-response curve represents the average and s.e. of three independent D4 assays. A 5-parameter logistic fit is shown by the blue curve. [76]

Table 2: Figures of merit (LOB, LOD, DR) calculated by averaging four independently generated dose-response curves for the detection of IL-6. Surface characterization using water contact angles (WTCA) and protein absorption levels.

<table>
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<tr>
<th>Surface</th>
<th>LOB (pg/mL)</th>
<th>LOD (pg/mL)</th>
<th>DR (log₁₀)</th>
<th>WTCA (Degrees)</th>
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</table>

2.2 Methods

2.2.1 Polymerization of PSBMA, PDMAEMA, and POEGMA on SiO₂ Surfaces

2.2.1.1. Functionalization of SiO₂ surfaces with ATRP initiator

All reagents were obtained from Sigma Aldrich (St. Louis, MO) unless stated otherwise. Glass slides (Schott Nexterion Glass B; Elmsford, NY) and silicon wafers with a thermally grown oxide layer (University Wafer, Inc; Boston, MA), were diced into 1x1 cm chips and were immersed in a 10% (v/v) solution of (3-aminopropyl)triethoxysilane (APTES) (Gelest, Inc.; Morrisville, PA) in 100% ethanol for 4 h. The substrates were extensively rinsed with ethanol and deionized (DI) water, centrifuged at 150 rcf in an
Allegra X-15R Centrifuge (Beckman Coulter; Indianapolis, IN) for 6 min, then cured overnight in an oven at 120°C. After cooling to room temperature, the substrates were immersed in a solution of 1% (v/v) α-bromoisoobutyryl bromide (BiB) and 1% (v/v) triethylamine in dichloromethane. Following incubation for 30 min, the substrates were removed from solution and consecutively rinsed with fresh dichloromethane, ethanol, and DI water. Finally, the substrates were centrifuged at 150 rcf for 6 min and cured at 120°C for 2 h, yielding surfaces functionalized with the ATRP initiator BiB (“APTES-BiB”).

2.2.1.2. Preparation of SBMA solution

Under an argon environment, a solution composed of 56 g of inhibitor-free sulfobetaine methacrylate (SBMA), 1.49 g of copper (II) bromide, and 9.21 mL of 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) was dissolved in 210 mL of methanol and 80 mL of DI water, and degassed by sparging with N₂ gas for 3 h. Next, 2.86 g of copper (I) bromide was slowly added to the solution under mild stirring until the salt was fully dissolved.

2.2.1.3. Preparation of DMAEMA solution

A solution composed of 150 g of 2-(dimethylamino)ethyl methacrylate (DMAEMA) (inhibitor-free), 28 mg of copper (II) bromide, 100 μL of HMTETA, and 250 mL of DI was degassed by sparging with helium for 3 h.
2.2.1.4. Preparation of POEGMA solution

A solution composed of 50 g of POEGMA (inhibitor-free), 28 mg of copper (II) bromide, 100 μL of HMTETA, and 350 mL of DI water was degassed by sparging with helium for 3 h.

2.2.1.5 Surface-initiated atom-transfer radical polymerization (SI-ATRP) of SBMA

Under an argon environment, the ATRP initiator-functionalized substrates (APTES-BiB) were incubated in the polymerization solution for a period of time which varied depending on the thickness required (Figure 8), and were extensively rinsed with DI water. The substrates were spun dry for 6 min at 150 rcf and stored under ambient conditions for subsequent use.

2.2.1.6 SI-ARGET-ATRP of DMAEMA and POEGMA

Surface-initiated activator regenerated by electron transfer atom-transfer radical polymerization (SI-ARGET-ATRP) was used to generate DMAEMA and POEGMA polymer brushes. Under an argon environment, 600 mg of sodium ascorbate was added to the polymerization solution previously described. The solution exhibited a color change from blue to violet indicating reduction of Cu(II) cations to Cu(I), and the substrates were immersed in the polymerization solution for a specified time that varied depending upon the thickness of the polymer brush required (Figure 13B). The substrates were rinsed extensively with DI water and were spun dry for 6 min at 150 rcf and stored under ambient conditions for subsequent use.
2.2.1.7 Conversion of PDMAEMA polymer brushes to PCBMA

A 20 mM solution of iodoacetic acid in 1X PBS was prepared and adjusted to a pH between 5 and 8 by dropwise addition of a 1 N NaOH solution. The PDMAEMA-coated substrates were incubated at 45°C or 75°C under mild stirring. The substrates were rinsed with DI water, spun dry for 6 min at 150 rcf, and stored under ambient conditions for subsequent use.

2.2.2 Surface characterization

2.2.2.1 Reflective mode ellipsometry

Film thickness was measured using an M-88 spectroscopic ellipsometer (J.A. Woollam Co; Lincoln, NE) at angles of 65°, 70°, and 75°, and wavelengths of 400–800 nm. To determine polymer brush thickness, a Cauchy model[86] with optical constants provided by the instrument’s software was used to minimize the mean squared error between the measured value of thickness and the thickness predicted by the model.

2.2.2.2 X-ray photoelectron spectroscopy

All substrates were rinsed with PBS at pH 9 and dried under a stream of N2 gas prior to being scanned. Data were acquired using an AXIS Ultra X-ray photoelectron spectrometer (Kratos Analytical, Inc., Chestnut Ridge, NJ). The spectrometer was operated at 15 kV and 10 mA with monochromatic AlK\textsubscript{x} x-rays (spot size: 700×400 μm). Survey scans were performed with 15 sweeps, a pass energy of 160 eV, an energy step of 1 eV, and a dwell time of 200 ms. High resolution scans were performed with 25 sweeps,
a pass energy of 20 eV, an energy step of 0.1 eV, and a dwell time of 269.7 ms. Operating pressure was ~1×10⁻⁸ torr. All spectral data were analyzed using CASA XPS software (Casa Software, Ltd.).

2.2.2.3 Drop contact angle measurements

Advancing sessile drop contact angles were measured using a Rame-Hart model 100-00 goniometer (Succasunna, NJ). Images were acquired immediately after a drop of DI water was placed on the surfaces, and contact angles were measured using Rame-Hart Drop Image Standard software.

2.2.2.4 Protein adsorption

Substrates were incubated for 24 h in a solution of bovine serum albumin conjugated to fluorescein isothiocyanate (FITC-BSA) at a concentration of 10 mg/mL in PBS. Following incubation, substrates were rinsed with 0.1% Tween-20 (v/v) in PBS three times with a BioTek ELx 5012 plate washer (Winooski, VT) programmed to perform an initial aspiration and add wash buffer at 300 μL.s⁻¹, while simultaneously aspirating the buffer at 300 μL.s⁻¹ with a height of 4 mm, to generate a mild stream of wash buffer to displace unbound material on the surface. This wash was followed by centrifugation for 5 s at 4800 rpm on a C1303 Slide Spinner (Labnet International; Edison, NJ) to remove excess liquid. Substrate were allowed to dry under ambient conditions. Fluorescence imaging was performed using an Axon Genepix 4400 tabletop scanner (Molecular
Devices; LLC, Sunnyvale, CA) under constant imaging conditions (photomultiplier gain, 400; excitation power, 100) and an excitation wavelength of 488 nm.

2.2.2.5 Anti-PEG binding assays

Substrates were incubated for 1 h in a 0.01 mg/mL solution of rabbit anti-PEG Ab (Thermo Fisher; Waltham, MA) in heat-inactivated calf serum (VWR; Radnor, PA). After rinsing three times with 0.1% Tween-20 (v/v) in PBS with a BioTek ELx 5012 plate washer as described above, surfaces were incubated for 1 h in a 2 μg/mL solution of Alexa Fluor 647-labeled anti-rabbit secondary Ab (Thermo Fisher; Waltham, MA) in PBS. Substrates were washed with 0.1% Tween-20 (v/v) in PBS using the BioTek ELx 5012 plate washer, spun dry at 4800 rpm on a C1303 Slide Spinner, and imaged on an Axon Genepix 4400 tabletop slide scanner under constant imaging conditions (photomultiplier gain, 750; excitation power, 100) and an excitation wavelength of 635 nm.

2.2.3 Immunoassay fabrication and performance

2.2.3.1 D4 immunoassay fabrication on polymer brush-coated surfaces

Lyophilized anti-IL-6 capture and detection Abs (R&D Systems; Minneapolis, MN) were reconstituted in sterile-filtered PBS to a final concentration of 1 mg/mL. Detection Abs were conjugated to Alexa Fluor 647, and trehalose was added to a final concentration of 1.0 % (w/v). Abc and fluorescently labeled Abd were printed on the substrate with a sciFLEXARRAYER S11 spotter (Scienion; Berlin, Germany). Capture spots were printed in a central array with detection spots in close proximity, but still
spatially separated from the central Ab. by hundreds of microns (Figure 3). After printing, the chips were left to dry and protected from light in a vacuum chamber lined with desiccant for 12 h under a mild vacuum of -25 in. Hg.

2.2.3.2 Dose-response curves

Dose-response curves were generated by exposing D4 microarrays to calf serum spiked with recombinant human IL-6 (R&D Systems; Minneapolis, MN) at different concentrations for 60 min. Following incubation, the microarrays were rinsed three times with 0.1% Tween-20 (v/v) in PBS with a BioTek ELx 5012 plate washer as described above. The substrates were spun dry with a C1303 Slide Spinner and arrays were imaged and quantified using an Axon Genepix 4400 (photomultiplier gain, 750; excitation power, 100). The limit of blank (LoB) was calculated by measuring the mean fluorescence intensity (μ) and standard deviation (σ) from 9 blank samples as \( \text{LoB} = \mu_{\text{blank}} + 1.645\sigma_{\text{blank}} \). The limit of detection (LoD) was calculated using low concentration samples (LCS) as \( \text{LoD} = \text{LoB} + 1.645\sigma_{\text{LCS}} \). The dynamic range (DR) was determined as the range of concentration from the LoD to the greatest concentration that had a fluorescent signal greater than 3σ of the next lowest concentration in the dilution series. All data was fit using a 5-parameter logistic curve using OriginPro 9.0 (OriginLab Corp; Northhampton, MA).

2.2.3.3 Imaging the D4 assay on POEGMA and PSBMA surfaces

Capture and detection spots inkjet-printed on the surface of PSBMA- and POEGMA-coated slides were imaged before addition of analyte using an Eclipse ME600
optical microscope (Nikon; Melville, NY) under 100X magnification in bright field imaging mode. After the assay, the surfaces were imaged using an Axon Genepix 4400 with a photomultiplier gain of 750 and excitation power of 100. Spot size was calculated with using GenePix Pro 7 software (Molecular Devices; Sunnyvale, CA).

### 2.2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, Inc; San Diego, CA). Student t-test, one-way ANOVA followed by Tukey’s post-hoc multiple comparisons test, and two-way ANOVA followed by Sidak’s post-hoc multiple comparison test were employed to evaluate the statistical significance of differences between groups. Specifically, Student t-test was used to evaluate the statistical significance of the water contact angles on PSBMA- and POEGMA-coated surfaces. One-way ANOVA followed by Tukey’s post-hoc test was used to evaluate the statistical significance of protein adsorption, anti-PEG Ab binding, and water contact angles between all surfaces. Two-way ANOVA followed by Sidak’s post-hoc test was used to evaluate the statistical significance of differences in the thickness of polymer brushes derivatized under different reaction conditions of pH and temperature, and differences between the spot diameters of capture and detection reagents printed on POEGMA and PSBMA brush-coated surfaces.
2.3 Conclusions

The D4 assay is a self-contained POCT based on the traditional sandwich immunoassay test format, but unlike other platforms that require extensive washes and incubation steps, the D4 assay has all reagents built-in-chip, which simplifies its use as little intervention and handling of the device by the user is required to complete the assay. Moreover, the assay manufacturing is simple as cAb and dAb are inkjet-printed directly onto the surface. This surface, to be useful for IVD device manufacturing, must simultaneously allow noncovalent biofunctionalization and protein resistance in its hydrated state. This somewhat incompatible behavior is naturally presented by POEGMA, which when dried, presents exceptional protein binding on its surface, enabling functionalization by inkjet printing, but when wet becomes protein resistant, allowing testing in complex milieu such as undiluted serum and whole blood.

Motivated by reports of the protein resistance of zwitterionic polymers brushes, we explored their use for fabrication of the D4 POCT. We found that although PSBMA zwitterionic brushes are nonfouling in their hydrated state, they are unsuitable for inkjet printing of antibodies in the dry state as they are too wettable, which translates to poor spot morphology and little noncovalent biofunctionalization. These results inspired us to develop a hybrid zwitterionic brush that enabled inkjet printing of antibodies in its dry state yet retained good protein resistance in its hydrated state. To do so, we developed a two-step synthesis in which an amine-functionalized brush of PDMAEMA was grown
from the surface by ATRP, followed by chemical derivatization of a fraction of the amine groups to generate a hybrid PCBMA-PDMAEMA brush. By controlling the extent of derivatization of the amine group to a carboxybetaine moiety, we created surfaces that satisfy both requirements. The hybrid polymer brushes were tested for detection of IL-6 in a D4-POCT, and showed analytical figures of merit comparable to the same assay fabricated on a POEGMA brush, with the advantage that they do not cross-react with anti-PEG Ab that compromise the utility of the D4 POCT on a POEGMA brush surface. This synthesis strategy provides a new class of polymer coatings for inkjet-printed point-of-care protein microarrays that exhibit a low detection limit and a high dynamic range. These results also shed light on the complex requirements for simple fabrication of protein microarrays.

Anti-PEG Abs can cause nonspecific interaction with POEGMA side chains. However, our previous work with clinical samples (Figure 7) demonstrated that antigen detection tests are less susceptible to this cross-reactivity than, for example, sequential Ab detection tests. The rationally-designed hybrid cationic-ZIB showed exceptional performance rivaling that of POEGMA-based D4, but the extra synthesis steps and fine tuning of the chemical group conversion extent adds undesired complexity to the assay fabrication. This loss in fabrication simplicity motivated us to pursue the application of the POEGMA-based D4 assay as the final platform for IVD test development, detailed in the chapters below.
3. A new approach for diagnosis of ebolavirus hemorrhagic fever

Our work with ebolavirus (EBOV) detection began during the 2014-2016 West Africa outbreak, the largest EBOV outbreak in history. During this epidemic, we devised a series of simple yet effective solutions to rapidly deliver a test that could rival the sensitivity of complex, costly, and time-consuming techniques such RT-PCR, with the ease of use of LFA-based rapid tests. Although these solutions were in an investigational stage and not deployed during the outbreak, they can now be translated into widespread use, increasing the level of preparedness of the scientific community to new outbreaks of Ebola or other infectious diseases.

The 2014-2016 West-Africa outbreak was marked by a lack of widespread diagnostic testing, especially in its initial stages, which hampered the establishment of quarantines and containment of potentially exposed individuals. Additionally, enactment of aggressive contact tracing and disinfection efforts throughout affected areas was impaired due to the significant delays in test results. During the height of the deadly outbreak, a laboratory diagnosis took on average 5 days to be released[87].

This challenging scenario drove us to embark on a multidisciplinary, long-term effort to reinvent the immunoassay development workflow for infectious diseases, employing the D4 Assay on POEGMA brushes as the enabling technology, and Zaire Ebolavirus (ZEBOV) as the prototypical pathogen, to rapidly deliver a low-cost, easy to
use IVD test targeting viral proteins with sufficient sensitivity to allow early detection of infection. This chapter presents the key initial steps of this project.

3.1 Zaire ebolavirus as target pathogen for IVD test development

The choice of ZEBOV as target pathogen for immunoassay development was driven by the risk associated with frequent disease outbreaks and the endemic status of this virus to several portions of the African continent. This negative-stranded RNA virus is transmitted to humans by close contact with blood, secretions, organs, or other bodily fluids of infected animals such as non-human primates, forest antelope, and fruit bats.

Since the discovery of EBOV in 1976, over 20 Ebola outbreaks have been recorded, with ~35,000 individuals infected and 15,000 casualties. Although different species of EBOV cause different clinical syndromes, fever, chills malaise, and myalgia are usually followed by multisystem involvement with prostration, anorexia, nausea, vomiting, and diarrhea, culminating in shock, convulsion, severe metabolic alterations, and deadly coagulation manifestations, which happen in ~50% of cases[88].

This high fatality rate, which can climb to 90% with ZEBOV, accompanied by frequent outbreaks in areas of the globe with poor healthcare infrastructure, renders this pathogen a dangerous public health menace and a potential biowarfare agent. Being able to rapidly identify infected individuals in areas with limited infrastructure is key to curb outbreaks.
3.1.1 Virology

EBOVs are part of the *Ebolavirus* genus and alongside Marburg viruses (MARV), form the Filoviridae family. Five species of EBOVs have been identified: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Bundibugyo ebolavirus* (BDBV), *Reston ebolavirus* (RESTV), and *Taï Forest ebolavirus* (TAFV). Only EBOV, BDBV, and SUDV have been associated with outbreaks in humans.

EBOVs are filamentous particles with ~80 nm diameter and variable lengths (Figure 20), and are composed of a ribonucleocapsid enveloped by a lipid membrane embedded with glycoprotein (GP). The EBOV’s negative-stranded RNA genome is composed of 19,000 nucleotides and seven genes encoding nucleoprotein (NP), glycoprotein (GP), RNA-dependent RNA polymerase (L), and viral proteins (VP) 24, 30, 35, and 40. All genes are monocistronic and encode for structural proteins except for the GP gene, which also encodes for a truncated version of the structural glycoprotein that is actively secreted from infected cells, named secreted glycoprotein (sGP).
Figure 20. TEM image of ZEBOV. Negative staining of viral particles adapted from Golding, C. G. et al. [89]

EBOVs (as other RNA viruses) present all elements required for initial viral transcription and replication in a host cell, namely an RNA genome coated by N protein, L, VP30 and VP35 proteins, contained in a ribonucleocapsid. Infection occurs by cell attachment through surface expressed proteins (lectins, integrins, TIM-1, Axl), followed by induced micropinocytosis and proteolytic cleavage of viral GP with cathepsin N and L. This cleavage exposes a Niemann-Pick C1 (NPC1) endosomal receptor binding domain which triggers viral and cellular membrane fusion. This fusion is followed by uncoating of the viral particle and transcription of RNA into mRNA[90]. Genome transcription is regulated by VP30 and VP35 complexed with protein L bound to the NP-coated genome and is followed by replication controlled through VP30 phosphorylation. Once replication starts, genome copies are coated with NP, VP24, VP30, and VP35 proteins. The
polymerase attaches to VP35 during assembly of the VP40 matrix, which drives the release of virions from the cell.

### 3.1.2 Epidemiology

Following the identification of filoviruses in 1967, hemorrhagic fever outbreaks were simultaneously reported in 1976 in Sudan and Zaire (now DRC), respectively caused by ZEBOV and SUDV[88]. These initial reports were followed by small outbreaks in isolated rural areas of sub-Saharan Africa and two larger ones, the first in Kikwit with 318 cases and the second with 425 cases in Gulu[91-93].

The rapid response aided by the World Health Organization (WHO) with contact tracing, isolation, and distribution of protective equipment for healthcare workers was paramount to curb both outbreaks. Interestingly, in Gulu, a field laboratory for sample testing was deployed for the first time and was important in diagnosing cases, aiding contact tracing and containment efforts.

The largest outbreak to-date occurred in West Africa and originated in Guinea, close to the border of Sierra Leone and Liberia. The first stage of the outbreak included 258 diagnosed patients in a 6-month period and was poorly contained, with the virus circulating openly in the border of the three countries. In early 2014, hundreds of individuals were infected at a funeral, which started the second and deadliest phase of the outbreak. This phase 2 presented an exponential growth of cases due to greater population density, larger mobility of people, and lack of control/containment measures,
and raised the concern of a pandemic. The third phase of the outbreak was marked by intense intervention of the international community with forced quarantines and aggressive contact tracing. At this stage, more efficient diagnostics greatly improved case management protocols, contact tracing, and containment measures.

Although the outbreak was considered over in late 2015, the ability of the virus to remain in immunologically privileged sites was responsible for re-emergences caused by sexual transmission. Similar to phase 3, this forth phase of the outbreak was marked by aggressive contact tracing and patient management. Phase 4 was also marked by extensive use of qRT-PCR and ring vaccination with experimental EBOV vaccines. Following the 2014-2016 outbreak, several cases of hemorrhagic fever were reported in the DRC in 2019. These initially isolated cases evolved into the ongoing 2019-2020 outbreak, the second largest ever recorded, with 3304 confirmed cases and a total of 2244 deaths in the Kivu province as of February 2020.

Investigation of index patients in outbreaks has indicated spillover events through consumption of virus-infected bushmeat or handling of infected animal carcasses. Although EBOV has not been isolated in bats in natural conditions, bats are thought to be the natural host of EBOV. Interestingly, in the 2007 Luebo (DRC) outbreak, there was strong evidence of transmission by contact with fruit bats[94], an event that was replayed in the 2014-2016 West Africa EBOV epidemic, where the initial case was traced to an 18-month-old boy believed to have been infected by bats[95].
Human-to-human transmission routes involve exposure to EBOV-infected bodily fluids such as saliva, sweat, blood, breast milk, stool samples, and semen through direct contact, fomites, droplets, and aerosols. Non-human primate models have shown that doses as small as 10 plaque-forming units of EBOV can cause ebolavirus disease, depending on the administration route, indicating the high infective potential of the virus and the importance aggressive containment and decontamination during outbreaks, efforts enabled by IVD testing of suspected cases.

3.1.3 Clinical manifestations

Early manifestations of EBOV infection are marked by high fever, myalgia, and malaise which start from 3 to 21 days following exposure to the virus. These initial symptoms are not specific to the disease, which poses a serious challenge for diagnosis as it is difficult to distinguish EBOV infection from other infections endemic to the region, such as typhoid fever and malaria. The initial symptoms of EBOV infection rapidly progress to anorexia, vomiting, nausea, abdominal pain, and copious diarrhea. Respiratory, vascular, and neurological manifestations are also common and frequently entail shortness of breath, chest pain, conjunctival injection, headache, confusion, delirium, and coma. Some patients recover following initial stages of the disease; evidence suggests that asymptomatic infections are rare but do occur. Most patients, especially those infected with ZEBOV (which has 90% lethality versus 50% for SUDV and 25% for BDBV), will progress to severe illness with hemorrhagic events which include
maculopapular rash, petechiae, mucosal hemorrhage, hematemesis, hematuria, and hematochezia (although hemorrhagic events were rare in the 2014-2016 West Africa outbreak). Later stages of the disease in acute patients is marked by shock, convulsions, and diffuse coagulopathy supervene. Acute EBOV is also marked by highly raised serum aminotransferases, creatinine, blood urea nitrogen, and proteinuria, which indicate hepatic and renal involvement. Thrombocytopenia, longer prothrombin times, and elevated D dimer levels indicate intravascular coagulation which precedes organ failure and shock.

Fatality is usually caused by hypovolemic shock and multiorgan failure and is related to elevated viral loads and early onset of symptoms. Non-fatal cases are marked by generation of specific IgM and IgG responses that are associated with an early and strong inflammatory response[96].

### 3.1.4 Case management

Without an approved treatment, case management involves isolation, supportive measures, and critical care when available. Severe dehydration and hypovolemia are at the core of organ and renal failure and must be treated with oral/intravenous fluids. In low-resource settings, case management also entails malaria treatment and broad-spectrum antibiotics, but confirmatory diagnosis should be prioritized. Patient isolation with barrier nursing protocols is the first step when managing a potentially infected patient. All healthcare workers must use appropriate personal protective equipment and
must be adequately trained to reduce personal risks and nosocomial exposures. Supportive care involves prevention or correction of fluid loss orally or intravenously. Adequate blood volume and electrolyte balance must be maintained to avoid organ and renal failure. Kidney and lung failure can be addressed using dialysis and extracorporeal membrane oxygenation in developed healthcare systems but are usually unavailable in low-resource settings[97].

3.1.5 Vaccine and experimental treatments

In the face of such a devastating illness, several efforts were focused on generating a protective vaccine[98]. Of the 15 vaccine candidates that were in pre-clinical stages, 8 were rapidly moved into clinical evaluation[99]. Of these initial candidates, conventional vaccines in which EBOV is heat- or chemically-inactivated were quickly discarded as they lacked effectiveness and risked reversion. Vector-based vaccines demonstrated partial protection with the exception of an adenovirus-based formulation which elicited full protection even in non-human primates.

In the race to generate a safe and effective vaccine formulation, a variation of an adenovirus-based vaccine, the rVSV-ZEBOV, was licensed and manufactured by Merck. The rVSV-ZEBOV is composed of a recombinant replication-competent stomatitis virus (VSV) in which one of the VSV genes is replaced by a gene that codes for the outer protein of the ZEBOV (VP40). This vaccine showed extensive protection and provided a tool to safely abate an outbreak when administered to individuals at risk of exposure to the
pathogen[100]. A randomized cluster vaccination clinical trial was performed with 3,537 contacts and contacts of contacts of EBOV-diagnosed patients receiving the vaccine immediately or after 21 days of potential exposure. The two arms were composed of 2,108 individuals of immediate and 1,429 of delayed vaccination. The results of this study were striking. The rVSV-ZEBOV vaccine resulted in 100% effective protection against ZEBOV infection. No EBOV disease was seen in the immediate vaccination group and only 10 cases occurred in the delayed vaccination study arm[101-102]. Safety studies conducted with 15,000 volunteers in Africa, Europe, and North America concluded that the vaccine was safe, and culminated in its use in the ongoing Kivu (DRC) outbreak and FDA approval in 2019 with the commercial name of Ervebo[103].

Although an Ebola vaccine is now available, further evaluations are required before healthcare bodies sanction its use outside ring vaccination protocols. Besides safety concerns, recent studies that estimated ZEBOV reproduction number (R₀, the average number of new cases an infected person will cause, which is 4 for ZEBOV) concluded that up to 80% of the population would need to be vaccinated to create herd immunity, a rate that would be challenging to reach in developing countries with large populations in rural areas and widespread mistrust of government healthcare policies[104]. The lack of trust towards the government was witnessed during the randomized cluster trial, as 34% of individuals that had been exposed to infected patients refused the vaccine. Another key hurdle to generating herd immunity is the cost of mass vaccinations, which is estimated
to be close to US$20 billion, a prohibitive sum for countries where the disease is endemic. Furthermore, there is still no evidence of long-term protection (>10 years) that would justify such an expensive prophylactic mass vaccination program.

In parallel to vaccine development, notable resources were allocated towards developing a treatment that could improve outcomes for infected patients. Throughout 2014-2016, several one-arm studies were conducted with experimental therapeutic agents under Emergency Use Authorization, but failed to yield scientifically-valid conclusions due to lack of appropriate controls[105-106]. A new drug candidate, ZMapp, which combined three GP-targeting monoclonal Abs, was evaluated when added to the standard of care (SOC) versus SOC alone in a randomized controlled trial in Guinea, Liberia, Sierra Leone, and the USA. Although a trend of improvement of survival was seen, the study outcome failed to meet statistical significance due to the small sample size, and with the end of the outbreak, no more patients could be enrolled[107].

During the ongoing DRC outbreak, the four most promising therapeutics under development were evaluated in the “PALM” clinical trial, in which all patients admitted to an Ebola Treatment Unit (ETU) were randomly assigned to receive Zmapp[107-108], mAb114[109-110], REGN-EB3[111-112], or remdesivir[113] in addition to optimized support care. Since ZMapp was the only drug that had been previously evaluated clinically, it was assigned as the control arm of the study[114]. After only 6 months and 681 patients enrolled, the trial was stopped as statistically significant results were
achieved which demonstrated superior outcomes for individuals treated with mAb114 or REGN-EB3 than those treated with ZMapp or remdesivir.

Patients treated with mAb114 and who showed recent onset of symptoms and low viral loads (signifying recent infection) had a 90% recovery rate. Patients with pronounced symptoms and high viral loads (representative of late stage infection) had only a 30% survival rate. These results demonstrate that early intervention is critical to improve treatment outcome, and underscore the importance of sensitive and widespread diagnosis to detect patients in early stages of the disease, to improve their chances of survival[114-115].

3.1.6 EBOV diagnostics

Widespread, rapid, reliable, and sensitive diagnosis is the most important step in curbing EBOV outbreaks. The out-of-control transmission rate seen in the 2014-2016 epidemic was greatly due to lack of testing infrastructure in affected countries[116]. In the beginning of 2014, only two laboratories in the region were fully equipped to handle patient samples, as qRT-PCR, the gold standard of EBOV testing, requires expensive equipment and trained personnel. RNA detection techniques are comprised of several steps that are challenging to implement: (1) sample collection, (2) viral inactivation, (3) viral RNA extraction, (4) reverse transcription, and (5) cDNA amplification. RNA extraction is tedious and labor-intensive and needs to be performed in a BSL-4 facility, but has been adapted to be performed in “hot laboratories”, increasing the risk of exposure to
laboratory personnel. RNA needs to be stored under refrigeration to avoid degradation and is prone to cross-contamination, causing inaccurate results.

Since 2014, several companies and research groups have worked to miniaturize and integrate qRT-PCR platforms so that these could be deployed in make-shift laboratories using small power supplies, but further technological developments are required to enable high-throughput capabilities and independence of large, complex equipment[117]. These efforts yielded the cartridge-based Xpert Ebola test, which runs on a modular GeneXpert equipment (Figure 21), which can process 1 to 16 samples simultaneously depending on the number of equipment modules. This automated system, developed by Cepheid, demonstrated sensitivity comparable to standard laboratory methods[118]. Importantly, the GeneXpert platform improved testing capabilities in Liberia and Guinea during the 2014-2016 outbreak by automating all qRT-PCR steps required for assay completion. Furthermore, their use of disposable cartridges encasing all reagents in the assay required only sample addition, simplifying training requirements and reducing risk of exposure. Although the testing platform played an important role in the targeted response during late stages of the West Africa epidemic and is currently being used in the Kivu outbreak, its widespread use is limited by the need for an auxiliary computer to control the testing unit, and a continuous energy supply to power the device. This need for infrastructure to house the equipment fails to address the need for a POCT that can rapidly deliver test results in low-resource settings.
The sensitivity and specificity of PCR-based techniques, which can detect the virus as early as 48 h following onset of symptoms, and the lack of sensitivity displayed by current immunoassays targeting viral antigens, have solidified the adoption of qRT-PCR as the gold standard for diagnosis of EBOV[120-121]. Although qRT-PCR is the recommended testing technique, its high cost, high complexity, lack of portability, and the risk of false-negative results caused by genetic drift[122] create an urgent demand for POC tests that effectively detect viral proteins in early stages of the disease.

Traditional EBOV immunoassays detect infection from 8 to 16 days after onset of symptoms, an unacceptably long time[123], especially considering that a patient’s infectivity increases with disease progression. In the aftermath of the West Africa outbreak, the WHO stressed the need for quicker tests at a lower cost. In response,
multiple LFAs were developed, including the FDA-approved Oraquick Ebola Rapid Antigen and the One-Step Ebola Test from Intec[124-126]. The LoD of the VP40-targeting OraQuick test is 53 ng/mL, which is comparable to the SERS nanotag platform (LoD = 66.1 ng/mL) that only detected infection at days 6-8 in non-human primate models with intramuscular (IM) infection and only when the viral load was high[127]. For comparison, standard qRT-PCR is able to accurately detect the virus as soon as day 3 following IM challenge[128-129]. The Intec test had up to 98% sensitivity in high viremia samples, but only 60% sensitivity in samples with low viral counts typical in the early stage of infection[125]. Clearly, LFAs for EBOV lack the sensitivity needed for early diagnosis, while the sensitive qRT-PCR assay lacks portability, deployability, and affordability. Therefore, there is a need for a new generation of diagnostics EBOV tests that can deliver the portability and ease-of-use of LFAs with the sensitivity and specificity of qRT-PCR.

3.2 Choice of sGP as EBOV diagnostic target for D4 assay

We believe that serological diagnostics have been limited due to the choice of biomarkers explored thus far in immunoassays. Throughout several outbreaks that occurred in the 1990s, ELISA was at the forefront of EBOV testing by detecting NP, VP40, and GP viral proteins. Although ELISA is easier to implement than qRT-PCR, the gain in performance and smaller immunological window of qRT-PCR justified its use in place of Ag detection tests.
Immunoassays targeting viral antigens have lacked the sensitivity required for early diagnostics, but the choice of a new IVD target related to EBOV disease combined with the ultra-sensitive D4 assay platform could elevate immunoassays to the forefront of the fight against EBOV infection. A D4 assay targeting secreted glycoprotein (sGP) could match or surpass qRT-PCR in sensitivity and provide diagnosis before onset of symptoms and contagion. This feat could curb outbreaks faster and save countless lives when combined with ring vaccination strategies and antibody-based drug therapies.

Our rationale for the use of sGP as a diagnostic target versus traditional structural proteins lies in the fact that active viremia—caused by viral replication in the host—happens in later stages of the infection, and it is only after viral particles are present in circulation that qRT-PCR is effective in detecting the infection. Notably, before active viremia, viruses must first highjack the cell machinery to express their proteins and synthesize and package their genetic material to build new viral particles. In most viral infections, these processes occur intracellularly, hidden from the humoral response of the host and from antibodies targeting these antigens in immunoassays. Similarly, qRT-PCR is only effective in diagnosing the disease after viral particles are released into circulation[130].

EBOV, unlike other viruses, actively expresses and secretes a truncated version of its transmembrane glycoprotein rapidly after cell infection (Figure 22). This process has
been demonstrated in vitro and we speculate that it also occurs in vivo in early stages of infection.

![Diagram of EBOV infection time course]

**Figure 22:** Proposed EBOV infection time course. (A-B) Viral particles infect host cells. (C) Infection is followed by secretion of sGP. (D) New viral particles are assembled and are released by the cell, starting active viremia.

The expression of GP, a protein responsible for fusion of the viral particle with the host cell, presents unusual regulatory mechanisms at the transcriptional and translational levels. Surprisingly, the default transcript of the glycoprotein gene is not the virion-associated GP, but rather sGP, with evidence suggesting that 80%[131] of the GP gene expression is the secreted version of the protein (Figure 23). Although the function of sGP has not been fully elucidated, this truncated version of GP is thought to be a key element in the subversion of the immune system, by providing a decoy target to the host’s humoral response[132-133]. Although reports on the time course and levels of sGP in circulation
during the infection are scarce, Sanchez et al.[134] demonstrated that this Ag is present in high amounts in the serum of acutely infected patients, which corroborates *in vitro* studies that demonstrate that sGP is secreted from the cell and expressed in higher titers than GP[135].

**Figure 23.** GP gene transcription profile. sGP is the main expression product and the structural GP1,2 results from RNA editing.

### 3.3 Generation of high efficiency sGP-targeting antibodies

D4 POCT performance depends on the quality of the antibodies integrated into the platform. Our previous work with commercially available antibodies yielded femtomolar LoDs and broad DRs with the D4 assay, supporting the goal of developing an immunoassay that matched qRT-PCR’s performance. Although monoclonal Abs have been generated to thousands of targets, we failed to identify a commercially available Ab pair targeting sGP. Consequently, our first step to create an EBOV D4 POCT was to develop sGP-targeting Abs.

To rapidly generate sensitive antibody candidates for sGP detection, we employed M13 phage display[136-137] coupled with chromatography-free Ab purification, followed
by surface plasmon resonance (SPR) to characterize and validate our Ab generation workflow.

### 3.3.1 scFv library generation

Phage-display and is a powerful technique for Ab generation\[138\]. The display of single chain variable fragments (scFV) on the surface of bacteriophage has several advantages versus hybridoma techniques, most importantly the greater number and diversity of potential binders generated and screened. Combinatorial reassortment can significantly increase the diversity of binders by joining one Ab’s heavy chain with another Ab’s light chain, generating binders that do not exist originally in the mice\[139\].

To develop sGP-targeting Abs, we partnered with Professor Michael D. Gunn’s laboratory, whose group performed Ag expression, mice immunizations, M13 phage-display library generation, and down-selection of sGP-targeting scFvs. To this end, mice were immunized with recombinant ZEBOV (Mayinga) sGP which was expressed in HEK 293 cells and purified using anti-HA tag Ab spin columns. To assess anti-sGP levels, Ag-down ELISA was performed by incubating dilutions of individual mouse serum in sGP-coated wells. The mice mounted a strong immune response following the immunization protocol (detailed in the Methods section), with Ab titers presenting significant ELISA readouts for dilutions of up to 1:409,600 (Figure 24A).

After additional immunizations to promote Ab affinity maturation, spleens from the mice with the highest titer immune responses were harvested and used to construct
an M13 library of scFv antibodies. mRNA from the spleen was reverse transcribed, followed by PCR to amplify the variable regions of the $V_H$ and $V_L$ chains. Next, equimolar amounts of the resulting $V_H$ and $V_L$ were pooled for splice end overlap to allow random association of heavy and light chains from all mice to increase combinatorial assortment. Following splice and overlap, the scFv inserts were cloned in a phagemid vector and electroporated into TG1 host cells to generate the scFv library.

Figure 24: Mouse IgG Titers for sGP and reactivity of individual scFv-phage clones for ZEBOV and SUDV sGP. (A) Mouse IgG serum titers for ZEBOV sGP. Ag-down ELISA for mice immunized with sGP preparations following terminal bleed. Each data point is the average and S.D. of measurements performed on 10 mice. (B) scFv-phage clone binding profile to ZEBOV after first round of positive selection evaluated using Ag-down ELISA. Each data point represents one scFV-phage binding
to ZEBOV sGP. (C) scFv-phage clone binding profile to ZEBOV and SUDV sGP following final rounds of positive selection, evaluated using Ag-down ELISA. Each data point pair (black and red) represents an scFv-phage clone with a distinct sequence.

This process was followed by phage expression and selection with sGP bound to immunotubes and a single round of positive selection. Following positive selection, individual clones were recovered and evaluated by Ag-down ELISA (Figure 24B). Next, in an attempt to refine the selection strategy and permit isolation of Abs binding to native conformational epitopes, phage selection was performed with sGP captured onto magnetic beads. This was performed using two rounds of positive selection with sGP from the EBOV (Zaire strain) and one negative sGP form EBOV Sudan, to demonstrate that highly specific binders could be isolated (Figure 24C).

3.3.2 Generation of scFv-Fc fusions

Following generation of scFvs targeting sGP, we identified 8 promising Ab candidates that could potentially be used in IVD applications. These candidates were sequenced and subcloned into a mammalian expression vector as mouse IgG2a (mIgG2a) Fc fusions. mIgG2a was specifically selected as the Fc fusion structure to facilitate downstream purification due to its strong affinity with protein A. We also employed the azurocidin (AZ) signal peptide to ensure appropriate cell trafficking and high secretion efficiency of the target protein[140].
Figure 25: pcDNA5 scFv-Fc expression vector design with cytomegalovirus (CMV) promoter and NheI, BamHI and XhoI cut sites. Mouse IgG2a Fc and azurocidin (AZ) signal peptide are also inserted in the vector.

Figure 25 depicts the strategy to generate scFv-Fc fusions. DNA containing the scFv-Fc sequences was cloned into PCDNA5 using Gibson Assembly[141], and transfected into Expi293 cells for expression. We harvested expression supernatant to purify the Ab candidates.

3.3.2.2 High-throughput purification of scFv-Fcs

To expedite scFv-Fc Ab purification, we employed a chromatography free IsoTag-based purification strategy (Figure 26). This process relies on Z-domain (ZD, the Fc-binding portion of protein A) fused to a thermally-responsive elastin-like polypeptide (ELP) that binds the Fc portion of the scFv-Fc and is purified out by a series centrifugation steps and salt-induced precipitations. Unlike protein A- or protein G-based chromatography, this method is low-cost, can be easily scaled up, and permits purification of multiple constructs simultaneously.

Affinity chromatography, which relies on protein A, protein G, or protein L immobilized to substrates, is the most popular method for purification of Fc-tagged molecules. Affinity chromatography is highly specific and enables rapid purification of
proteins in few steps. However, column-based affinity chromatography is costly, requires harsh elution conditions which can irreversibly damage the product, and has limited scalability and volume output.

Figure 26: Illustration of chromatography-free, IsoTag-based, Fc-fusion purification strategy. IsoTag (ZD-ELP) is added to the cell culture extract and binds mIgG2a Fc. A cold-spin followed by collection of the supernatant are performed to isolate soluble proteins from cellular debris and precipitates. 0.4 M ammonium sulfate is added triggering phase-transition of the ZD-ELP bound to the Fc-fusion protein, which becomes insoluble. Following centrifugation, the supernatant is discarded and the pellet is resuspended in PBS. This process can be repeated multiple times until the desired purity level is achieved. The pellet is resuspended in sodium citrate buffer at pH 4. Finally, a room temperature spin is performed after ammonium sulfate is added and the supernatant contains the purified Fc fusion protein.
To circumvent these limitations, affinity precipitation methods have become a viable option, as purification is based on a simple environmental trigger and the specificity of Ab binding proteins[142]. Initial demonstrations employing thermally-responsive elastin-like-polypeptides (ELPs) fused to Ab-binding proteins have shown promising results. The extensive characterization of ELPs (a class of stimuli-responsive biopolymer inspired by intrinsically disordered domains of tropoelastin) grants a wealth of knowledge regarding their behavior and properties[143-144]. Their thermally-triggered lower critical solution temperature (LCST) phase transition has been extensively used for protein purification, as the ELPs aggregate and become insoluble when heated above a critical temperature, and turn soluble once cooled, a behavior that is opposite that of other proteins and can be used for protein purification by using a series of cold and hot spins. Another key feature of ELPs is that the transition temperature can be lowered by the addition of salt, which reduces the need to increase solution temperature, as some proteins are thermally sensitive and may degrade if heated.

We performed chromatography-free Ab purification (Figure 26) of the eight Ab candidates by employing a 20% alanine ELP-ZD fusion (37 kDa) expressed in E. coli and purified through phase transition (Figure 27A), which demonstrated reversible phase behavior (Figure 27B).
Figure 27: Isotag purification and phase behavior. (A) SDS-PAGE of a 20% alanine ELP-ZD (37 kDa), where lane 1 is the pre-purification cell lysate, lane 2 is the supernatant post polyethyleneimine (PEI) precipitation, and lane 3 the supernatant after phase-transition and hot spin. Lanes 4, 6, and 8 show the supernatant of cold spin. Lanes 5 and 7 show the supernatant of hot spins following salt-triggered phase-transition. (B) ELP-ZD phase transition characterization using turbidity as a function of temperature and concentration in solution.

The purification process entailed the addition of a molar excess of ZD-ELP to the expression flasks followed by a cold spin to remove cellular debris. We triggered phase transition through salt addition and performed a room temperature spin. The resulting supernatant was discarded and the pellet containing ELP-ZD and Abs was resuspended in PBS. Following a cold spin, the supernatant was separated and transitioned with salt addition. This was followed by a hot spin cycle with removal of supernatant and resuspension of the pellet in a sodium ascorbate pH 4 buffer to break the bond between the ELP-ZD and Abs. After a final round of salt-induced phase transition and centrifugation, the Abs in the supernatant were isolated from the pelleted ELP-ZD.
The outcome of each step of this process for one of the scFv-Fcs is shown in the SDS-PAGE gel in Figure 28A, which shows an effective Ab purification. Figure 28B shows another SDS-PAGE gel with the final purification product for all scFv-Fcs.

Following purification, we evaluated the purified scFv-Fc binding towards ZEBOV sGP. We initially ran Ag-down ELISA tests (Figure 28C). Of the eight initial constructs, one did not present a detectable expression level. A1B7-1 scFv-Fc expression yields were at 20–50-fold lower than for other Ab candidates, which inhibits scaling up efforts and is indicative of a lack of stability, undesirable in a POCT antibody.

Figure 28: Isotag based scFv-Fc purification. (A) Isolation of A1F3-1 scFv-Fc using IsoTag purification, run on an SDS-PAGE gel. Lanes 1-6: (1) cell culture extract prior to addition of IsoTag, (2) cell culture extract after addition of IsoTag, (3) cold-spin supernatant, (4) room temperature spin supernatant, (5) resuspended pellet following an extra round of cold-spin, (6) purified scFv-Fc construct run under reducing conditions. IsoTag is marked by a blue arrow and purified scFv-Fc is shown by a red arrow. (B) Purified scFv-Fc fusions run on an SDS-PAGE gel. Lanes 1 and 2 for each construct show purified scFv-Fc under (1) reducing and (2) non-reducing
3.3.3 Characterization of Ab candidates using SPR

To identify the best antibodies for IVD applications (those with the highest affinity), we measured candidate scFv-Fc binding constants ($K_D$, equilibrium dissociation constant) using surface plasmon resonance (SPR)[145-146]. Employing a Protein A SPR chip and a Biacore T200, we determined the scFv-Fc binding constants by fitting the sensorgram curves with a 1:1 binding model. The sensorgram for the Ab candidates with multiple Ag concentrations can be seen in Figure 29, and the average binding constants of three independent runs for each scFv-Fc are summarized in Table 3.

High affinity antibodies have $K_D$ values between $10^{-8}$ and $10^{-12}$. All of our generated antibodies displayed $K_D = \sim 10^{-8}$ except for A1F3-1, with an affinity ~100-fold higher than the other constructs. All generated Abs possessed low nM to high pM affinities, promising for downstream application in IVD test development.
Figure 29: Binding constant determination of scFv-Fcs. Sensograms of (A) C2bA2-2, (B) A1B4-1, (C) A1F3-1, (D) A1F5-1, (E) C2bA5-2, and (F) C2B6-2. scFv-Fcs were bound to Protein A chips and different concentrations of ZEBOV sGP were injected. Binding constants were determined using global fits and local Rmax fit of three independent sensograms based on the 1:1 reaction model. KD = Equilibrium dissociation constant. Each data point represents the average and standard error of three independent measurements.

Table 3: Binding constants of scFv-Fc Ab candidates. Each data point represents the average and standard error of three independent measurements. Kon = Association constant; Koff = Dissociation constant and KD = Equilibrium dissociation constant.
3.4 Methods

3.4.1 ZEBOV sGP expression and purification

A ZEBOV sGP expression plasmid consisting of ZEBOV sGP (Mayinga) with an N-terminal HA tag under control of the CMV promoter in pDISPLAY vector (PMID: 15681442) was provided by Dr. Thomas Hoenen at Rocky Mountain Laboratories. *E. coli* containing the plasmids for each ZEBOV sGP were cultured overnight in Luria broth with ampicillin at 50 μg/mL (Calbiochem). Plasmid DNA was purified using a Plasmid Plus Midi Kit (Genesee; USA). Purified DNA was Sanger sequenced (Eton Bioscience) and transfected into Expi 293 cells (Thermo Fisher) with Expifectamine. ZEBOV sGP protein was purified from culture supernatants by anti-HA-tag affinity chromatography (Thermo Fischer) and stored at -80°C in 1x PBS.

3.4.2 M13 phage display library generation

3.4.2.1. Immunizations and sera harvest

Five WT BALB/c mice and five WT C57BL/6 mice (Charles River Laboratories) were immunized intraperitoneally with 100 mg ZEBOV sGP in a 50% slurry of alum. The mice were boosted with 50 mg ZEBOV sGP/alum intraperitoneally at 14-day intervals. Mice were periodically bled to obtain serum to assay anti-ZEBOV sGP IgG titers by Ag-down ELISA. ELISA measurements were performed as previously described[137] using 1 μg/mL sGP diluted in PBS as coating antigen. After the anti-sGP immune titers reached
plateau, a final boost was administered. Spleens were harvested 4 days after terminal boost and homogenized into Trizol (Life Technologies).

3.4.2.2. scFv library construction

RNA was prepared from harvested spleens and reverse transcribed with random hexamers as previously described[137]. Sets of gene-specific primers (PMID:9032408) were used to amplify \( V_H \) and \( V_L \) fragments. Splice extension overlap was used to join \( VH \) and \( VL \) fragments with an intervening flexible linker, \((G4S)_3\), and to append restriction sites for ligation into the phage display vector pCANTAB5E. Following ligation, 30 electroporations of \( F' \) \( E. \) \( coli \) strain TG-1 (Lucigen) were pooled and plated on 2xYT plates supplemented with glucose (2%) and ampicillin (0.1 mg/mL). The resulting colonies were scraped into 2xYT and pooled to create library bacterial stocks from which M13 phage particles were generated by M13 K07 (New England Biolabs, USA) rescue for selections.

3.4.2.3. Phage rescue

As described elsewhere in greater detail[137, 147], rescues with M13K07 were performed to obtain M13 phage particles for selections and screening. A 100 mL 2xYT/AG culture was inoculated from frozen stocks and grown at 37°C to an OD600 of 0.7. M13K07 helper phage \((2 \times 10^{11})\) were added and the culture was shaken at 200 rpm for 30 min at 37°C. Bacteria were pelleted and the medium replaced with 50 mL of 2xYT supplemented with ampicillin and kanamycin. The culture was shaken at 37°C for 30 min, then at 30°C for 16 h. The culture was centrifuged as above, and the supernatant was subjected to
multiple precipitations in 0.2 V of ice-cold 20% polyethylene glycol 8000 / 2.5 M NaCl to purify and concentrate the phage particles which were pelleted. Following the last precipitation, the phage were resuspended in 1 ml PBS and stored at 4°C. Phage titers were determined by infections of TG-1 cells with serially diluted phage.

Individual phage clones were rescued for screening in sterile 2 mL/well 96-well plates (Continental Lab Products). 400 μL of 2xYT/AG was inoculated with a phage clone and shaken at 200 rpm for 16 h at 30°C. Aliquots from the wells were used to inoculate a rescue plate containing 400 μL of 2xYT/AG supplemented with 2×10¹⁰ phage/mL of M13K07 helper phage. The rescue plate was shaken at 37°C for 4 h. Bacteria were pelleted at 3000g for 10 min, resuspended in 400 μL of 2xYT/AK, and shaken for 16 h at 30°C. The bacteria were pelleted as above and the supernatant containing phage particles was transferred to a fresh plate for screening assays.

### 3.4.3 Immunotube selection

Recombinant ZEBOV sGP (50 μg in 1 mL PBS) was adsorbed to NUNC immunotubes. Following incubation, this tube and a control tube were blocked with 4 mL of 2% milk in PBS. An aliquot of 1011 freshly prepared M13 phage particles from the sGP scFv library was precleared for 1 h at room temperature in the milk in PBS control tube then transferred to the target tube with sGP for a 1 h incubation. Unbound phage were removed with 20 washes of 4 mL PBS with 0.1% Tween-20 followed by 20 washes with 4 mL PBS. A 1 mL aliquot of log-phase TG-1 bacteria was added to the target tube and
incubated at 37°C for 30 min to allow infection with the bound phage. The TG-1 cells were plated on 2xYT/AG plates and grown overnight at 30°C. Individual phagemid colonies were rescued in 96-well plates to produce phage particles for screens as detailed above.

3.4.4 Generation of scFv-Fc constructs

Sequences of eight scFvs selected using Ag-down ELISA were provided by Assistant Professor of Medicine Barbara Lipes. The eight scFvs were subcloned into pcDNA5 vectors (Thermo Fisher) for expression in a mammalian system. To this end, E. coli with pcDNA5 plasmid was grown in ampicillin-rich Terrific Broth (TB) media (MoBiolabs), and plasmid was isolated using a QIAprep Spin Miniprep Kit (Qiagen). pcDNA5 plasmid was cut with NheI and XhoI (New England Biolabs) and gel purified. A gBlock DNA fragment (Integrated DNA Technology) with mouse IgG2a sequence and appropriate overhangs was inserted into the pcDNA5 vector using Gibson Assembly Master Mix (New England Biolabs). The resulting construct was transformed into NEB5α cells (InvitroGen) which were recovered with SOC media (Sigma Aldrich) for 60 min at 37°C, and later selected in TB agar plates with 50 μg/mL ampicillin (Calbiochem). E. Coli with pcDNA5 vector with Fc insert was grown and isolated as previously described. pcDNA5-Fc was Sanger sequenced using appropriate primers (Genewiz), cut with NheI and BamHI, and gel purified. gBlocks containing the eight scFv sequences were inserted into the Fc-pcDNA5 construct using Gibson assembly. The resulting constructs were transformed into NEB5α cells and plated in ampicillin TB/agar plates.
pcDNA5 vector with scFv-Fc inserts were grown in ampicillin-rich TB media and the plasmids were isolated using a QIAprep Spin Miniprep Kit and Sanger sequenced.

3.4.5 Expression of scFv-Fc antibodies

scFv-Fc antibody expression was performed using an Expi293 high efficiency transient expression system (Thermo Fisher). Isolated and sequenced plasmids for each of the scFv-Fc fusions were retransformed into NEB5α cells and cultured overnight in ampicillin TB/agar plates. Isolated colonies were grown in ampicillin-rich TB, and DNA plasmid was purified using a QIAprep Spin Midiprep Plus Kit. Purified DNA was Sanger sequenced to guarantee correct expression products and transfected into Expi293 cells.

3.4.6 IsoTag purification of scFv-Fc antibodies and IgGs

Expression flasks received 3.5 µM of IsoTag per mL of culture and were incubated for 10 min at 37°C. Expression products were centrifuged at 14,000 rpm for 10 min at 4°C to remove cells and cellular debris. IsoTag-bound Abs were purified from culture supernatant by two rounds of inverse thermal cycling[148]. To avoid prolonged heat exposure, Isotag-bound Abs were phase-separated by addition of 0.5 M ammonium sulfate and centrifuged at 14,000 rpm at 37°C for 10 min. Pellets were resuspended in 1x PBS and centrifuged at 14,000 rpm at 4°C for 10 min. Following a second round of thermal cycling, the pellet was resuspended in 50 mM citric acid buffer pH 4, and 0.5 M ammonium sulfate was added to trigger phase transition. The Ab-rich supernatant was separated from the IsoTag pellet and neutralized with 0.1 mL of 1 M Tris pH 8.5 per mL
of supernatant. Isolated Abs were buffer exchanged with 10K Amicon centrifugal devices and concentrated to 1 mg/mL solutions in PBS.

### 3.4.7 Binding kinetics of scFv-Fcs to ZEBOV sGP

Binding kinetics were determined by SPR using a Biacore T200 system (GE Healthcare). scFv-Fc solutions at 1 μg/mL in HBS-EP running buffer (GE Healthcare) were injected into a protein A sensor chip at 5 μL/min until a final RU of ~350 was reached. For scFv-Fcs, different concentrations of ZEBOV sGP were prepared using a 2-fold dilution series in HBS-EP running buffer and were injected at 30 μL/min for 200 s; dissociation was monitored for 600 s. Following each injection round of scFv-Fc and sGP, the protein A chip surface was regenerated using a double 30 s injection of 10 mM of pH 2 glycine-HCl buffer at 30 μL/min. Experimental SPR data for Ab/Ag interactions were fit using a 1:1 binding model using global fits and local Rmax with BIA evaluation software (GE Healthcare).

### 3.5 Conclusions

Early detection of EBOV is critical in efforts to curb disease outbreaks. Recent developments in vaccines and therapeutics geared towards EBOV disease have not been accompanied by novel IVD test approaches for widespread rapid population testing. EBOV detection still relies on qRT-PCR-based techniques, which are high-cost, non-portable, and lack ease of use. Current immunoassays are easy to use and fabricate, but lack the sensitivity of qRT-PCR. To challenge this paradigm, we generated a D4 assay to
detect a novel EBOV biomarker, the truncated and actively secreted version of GP, sGP. This protein has been overlooked as a diagnostic target, but recent studies indicate that this biomarker could be present in circulation in early stages of the infection in amounts that could be easily detected by the D4 assay.

Based on this hypothesis, we generated sGP-targeting scFv Ab candidates by employing M13 phage display and iterative enrichment protocols. This process resulted in eight candidates with promising binding to sGP, which were converted into Fc fusions for expression in mammalian systems. To address a key challenge with IVD development—purification of multiple target Abs for downstream applications—we employed a chromatography-free purification strategy based on thermally-responsive ELPs fused to Ab-binding molecules. This approach enabled parallelization and increased process throughput, addressing the bottleneck that Ab purification may pose to test development. This initial Ab development endeavor laid the foundation for the development of the ZEBOV D4 assay, presented below.
4. A new method to select antibodies for IVD tests

Sandwich immunoassay test development traditionally relies on the availability of Abs that bind non-overlapping epitopes of a target antigen, but the process of generating Ab pairs suitable for IVD applications using high-throughput screening techniques such as phage display is costly, time-consuming, and often yields suboptimal results.

Following expression of the selected sGP Ab candidates as scFv-Fcs and characterization by SPR, we were faced with the challenge of assessing the performance of the scFv-Fcs as capture and detection reagents. This process, which for six candidates (which showed high expression and high binding affinity) would require thousands of ELISA wells and weeks of work to identify effective capture and detection Ab pairs, initially seemed impractically long and expensive[149]. On the other hand, not screening potential pairs may result in suboptimal reagents and an IVD test with poor performance.

Another approach to identify antibody pairs relies on SPR[150]. In addition to binding kinetics studies, SPR has also been used in epitope mapping of proteins, with the end goal of structural analysis and characterization of antibody binding sites. The main advantage of SPR over ELISA lies in the real-time measurement of binding, which relies on a label-free approach. But similar to ELISA, low throughput in SPR can be an issue if large numbers of reagent combinations need to be evaluated. The need to sequentially
inject reagents and the limited number of flow-cells in an SPR chip may also cause long
and complex test runs.

The low throughput of SPR has been partially addressed with the use of array-
based systems. But these platforms, which can process multiple Abs simultaneously, are
costly and require complex software to operate and perform data analysis. Multiplex SPR
chips are also susceptible to nonspecific protein binding, limiting its broader use [151].

Faced with the challenge of identifying the optimal Ab pair for the ZEBOV D4 assay, we envisioned a simple, fast, and cost-efficient solution: the use of the D4 assay as
a tool for high-throughput Ab pairing.

4.1 The D4 assay as a tool for antibody pairing

This screening strategy, which leverages the fact that the D4 assay is in its core a
protein microarray with extensive multiplexing capabilities, entails the noncovalent
functionalization of a POEGMA-coated chip by inkjet-printing all of the reagents as cAbs
in the center portion of the chip (Figure 30). These cAbs are printed as rows of discrete
spots where each row contains a different cAb. This step is immediately followed by printing one of the Ab candidates as a FL-dAb in a 10% trehalose solution.

Figure 30. Ab screening strategy using the D4 assay as a high-throughput pairing tool to determine which binders can be used as capture and detection reagents. i) Fabrication strategy, in which all binders are printed in a central portion of the chip as capture spots. Rings of one fluorescently-labelled scFv-Fc are printed around the capture spots pre-mixed with a 10% trehalose solution. ii) Analyte-spiked FBS was added and, following a final wash step, fluorescence output was read and quantified. iii) Fluorescence output is used to obtain dose-response curves of all reagents for capture, with one of the reagents for detection.

Following chip fabrication, the assays are incubated with different concentrations of target analyte spiked in FBS. Following a wash step, the spot fluorescence can be imaged and quantified using a tabletop scanner.

By employing this strategy, we envisioned that the dose-response behavior of all the combinations of Abs as capture and detection reagents could be quantified, and the optimal pair determined based on LoDs. This functional screening approach could enable
the simultaneous analysis of dozens of Abs already in the platform of the final ZEBOV D4 assay, obviating the need for extensive optimization required by other IVD tests.

4.2 Identifying the optimal antibodies for EBOV detection

Employing this Ab pairing scheme (Figure 30), we inkjet-printed each of the scFVs as capture reagent on POEGMA-coated slides. Next, we printed one Alexa Fluor 647-labelled scFV-FC with trehalose in concentric rings around the capture spots. Finally, we incubated each assay with different concentrations of sGP spiked in FBS for 90 min, and following a wash step we scanned the chips using a GenePix 4400 microarray scanner to quantify the capture spot fluorescence output. The calculated LoDs and DRs for all combinations of Abs are listed in Table 1, and the dose-response curves are shown in Figure 31. Most of the reagents do not function as pairs although they target sGP, as previously confirmed by Ag-down ELISA. Only a few constructs presented dose-dependent behavior, in which the fluorescence intensity increased with increasing Ag concentration added to the chip.

We identified one construct that performed exceptionally well as capture reagent: A1F3-1, which showed a $K_D$ of $\sim10^{-10}$, due to a higher association constant ($K_{on}$) than the other scFv-Fcs. ($K_{off}$ values were similar between the constructs.) A1F3-1 paired effectively with all of the other scFV-FCs used as detection antibodies. By analyzing all compiled LoDs and DRs, we identified A1F3-1 as cAb and C2bA5-2 as dAb.
This approach allowed the straightforward identification of antibody pairs for use as capture and detection probes, as can be seen in the dose-response curves of C2bA5-2 as cAb and A1F3-1 as dAb (Figure 31). We also observed that suboptimal pairs could be mistakenly selected if all possible combinations were not simultaneously tested. These results demonstrated that this approach could generate useful data for identifying Ab candidates for IVD test development.

Table 4: LoD and DR values for each Ab pair. “-” indicates no dose-response curve behavior. LoD = limit of detection; DR = dynamic range.

<table>
<thead>
<tr>
<th>scFv-Fc Pairing in D4 Assay</th>
<th>C2bA2-2</th>
<th>A1B4-1</th>
<th>A1F3-1</th>
<th>A1F5-1</th>
<th>C2bA5-2</th>
<th>C2B6-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection</td>
<td>LoD (ng/mL)(log10)</td>
<td>DR (ng/mL)(log10)</td>
<td>LoD (ng/mL)(log10)</td>
<td>DR (ng/mL)(log10)</td>
<td>LoD (ng/mL)(log10)</td>
<td>DR (ng/mL)(log10)</td>
</tr>
<tr>
<td>Capture</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.34</td>
<td>1.77</td>
</tr>
<tr>
<td>C2bA2-2</td>
<td>0.31</td>
<td>3.2</td>
<td>0.24</td>
<td>3.3</td>
<td>30.1</td>
<td>1.22</td>
</tr>
<tr>
<td>A1F3-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.21</td>
<td>2.2</td>
</tr>
<tr>
<td>A1F5-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.58</td>
<td>1.88</td>
</tr>
<tr>
<td>C2bA5-2</td>
<td>1.35</td>
<td>2.6</td>
<td>72.64</td>
<td>0.8</td>
<td>72.64</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 31. Antibody pairing dose-response curves. (A-F) Dose-response curves of C2bA2-2, A1B4-1, A1F3-1, A1F5-1, C2bA5-2, and C2B6-2 scFv-Fcs evaluated as capture reagent versus fluorescently-labelled C2bA2-2 (A), A1B4-1 (B), A1F3-1 (C), A1F5-1 (D), C2bA5-2 (E), and C2B6-2 (F) as detection reagent. Each data point represents the average and standard error of four independent D4 assays. Dose-response highlighted in black indicates A1F3-1 as capture reagent in all panels. Dose-response curves were fitted with a 5-parameter logistic fit.
4.3 SPR-based antibody pairing

We next compared the antibody-pairing capability of the D4 assay with that other standard techniques for antibody screening. We selected SPR for evaluation due to its widespread use for epitope-mapping employing two or more Abs[150].

Of the experimental schemes that can be used with SPR, the strategy which best simulates an immunoassay requires immobilization of one Ab candidate on the surface, which is then exposed to Ag and a second Ab. The challenges with this approach are the need for multiple SPR chips (which are costly) and the need to optimize conjugation conditions (concentration, flow rate, and flow time of the Ab on NHS-activated surfaces)[152]. These technical requirements are a barrier to the frequent use of this approach.

An alternative approach is immobilization of Ag on the chip followed by sequential flow of Abs. This approach can provide valuable information regarding binding site availability to multiple Abs, and involves covalent immobilization of Ag on the chip, which is exposed to one Ab until all epitopes for that binder are covered (indicated by a plateau in the sensorgram). Next, the Ag is exposed to the second Ab; if there is a significant increase in SPR response units (RU), we can conclude that these Abs target non-overlapping regions of the Ag, and that this Ab pair is a candidate for use in IVD test development. If there is no increase in RU when flowing the second Ab, this indicates that the two Ab target the same epitope or the first Ab blocks the second epitope,
and the Ab pair is not suitable for IVD application. This strategy is advantageous because it enables evaluation of multiple Abs using a single chip, eliminating the need for optimization of probe density on the gold-coated surface.

Using this competitive binding strategy, we tested all Ab combinations by injecting one Ab in the flow cell, followed by a stabilization period during which only buffer was flowed through the system. The stabilization period was followed by a second Ab injection and a final regeneration step. The results from this approach, shown in Figure 32, were unexpected in several ways. First, most Ab pairs were deemed compatible, a result that was not reproduced in the D4 screening step, in which most of the Ab pairs showed no dose-dependent behavior. Second and more importantly, the A1F3-1 scFv-Fc elicited the smallest RU although it had the highest binding affinity based on the previous D4 assay analysis. We speculate that this surprisingly low RU for A1F3-1 but high binding affinity using the D4 assay may have been due to blockage of the A1F3-1 binding epitope of sGP during sGP immobilization: A1F3-1 may target the lysine-rich region of sGP that is covalently immobilized to the chip surface via NHS-ester chemistry.

Importantly, the technique indicated that C2bA5-2 was the most compatible Ab for A1F3-1, consistent with the D4 assay.
Figure 32: Sensorgrams of competitive binding Ab mapping. sGP was covalently bound on chip surface and one scFv-Fc was passed through the flow cell, followed by a stabilization interval and injection of the second scFv-Fc.

These results demonstrated the value of the D4 assay as an Ab pairing platform. SPR uses large quantities of expensive antibody reagents, is labor intensive, and is time consuming, with much lower throughput than the D4 assay. The D4 assay may be a revolutionary platform that represents not only a simpler IVD test format but also a means for selection of antibody pairs. Furthermore, it is likely that the A1F3-1–C2bA5-2 pair would not have been identified if the D4 assay-based pairing strategy had not been devised.
4.5 Methods

4.5.1 D4 assay-enabled high-throughput antibody pairing

Following fabrication (described in Section 2.3.3)[57, 76], down-selected scFv-Fc Abs were conjugated to Alexa Fluor 647 and diluted with a trehalose solution in 1x PBS to a final concentration of 10% (w/v) trehalose, 0.25 mg/mL Ab. Aliquots of scFv-Fcs to be tested as cAb had trehalose added to a concentration of 0.05% (w/v) in 1x PBS. cAbs were printed in the central area of the chip in close proximity, and one FL-dAb in trehalose was also non-contact dispensed in a concentric pattern around the central area of the chip using a sciFLEXARRAYER S11 spotter (Scienion). After printing, the chips were left to dry and protected from light in a vacuum chamber (−25 in. Hg) lined with desiccant for 1 h.

After fabrication, dose–response curves were generated by exposing D4 microarrays to FBS (Avantor) spiked with ZEBOV sGP at different concentrations for 90 min. Each point of the curve is the mean and standard error of four replicates. Following incubation, the chips were rinsed with 0.1% Tween-20 (v/v) in 1x PBS and spun dry using a C1303 slide spinner (Labnet International).

Arrays were imaged and quantified using an Axon Genepix 4400 (Molecular Devices) (photomultiplier gain, 750; excitation power, 100). LoDs were determined by using low-concentration samples (LCS) and the mathematical formula LoD = LoB + 1.645σLCS, where the limit of blank (LoB) was determined by measuring the mean fluorescence intensity (μ) and standard deviation (σ) from twelve blank samples and
using the definition \( \text{LoB} = \mu_{\text{blank}} + 1.645\sigma_{\text{blank}} \). Dynamic range (DR) was determined as the range of concentration from the LoD to the greatest concentration that had a fluorescent signal greater than \( 3\sigma \) of the next lowest concentration in the dilution series. Data was fit using a five-parameter logistic curve using OriginPro 9.0.

### 4.5.2 SPR-based epitope mapping through competitive binding

Epitope mapping was performed using SPR with a Biacore T200 system. sGP was covalently bound to CM5 chips via amine groups until an RU of \(~450\) was reached. Sensorgrams were then generated by sequential injection of two antibodies at 30 µL/mL for 800 s separated by a 200 s stabilization period. Chips were regenerated using 10 mM glycine buffer pH 2.0. scFv-Fcs at 1.0 mg/mL in PBS were diluted using HBS-EP to 10 µg/mL before injection.

### 4.6 Conclusions

Following scFv-Fc expression, purification, and affinity characterization, we identified Ab pairs that could be integrated into the D4 assay, with the end goal of generating a POCT for EBOV detection. For the first time, we demonstrated how the D4’s multiplexing capabilities can be leveraged in a high-throughput Ab pairing strategy which rapidly yields optimal pairs of capture and detection antibodies for IVD applications. We compared this new approach to SPR-based epitope-mapping, and found that the D4 assay Ab-pairing method allowed identification of an effective Ab pair that may not have been identified using SPR.
This simple and elegant Ab pairing strategy addresses the bottleneck in IVD assay development of identifying optimal capture and detection reagents. This Ab pairing approach may be the greatest contribution of this work to the field of IVD, as Ab pairing has become an indispensable asset in the D4 assay development toolbox. The D4 assay Ab pairing method integrates seamlessly into the Ab development workflow, and when combined with scFv phage display for rapid Ab generation and chromatography-free Ab purification, may significantly shorten the time required to generate an Ag-targeting sandwich immunoassay.

In the next chapter we describe Marburg virus cross-reactivity studies using the A1F3-1–C2bA5-2 scFv-Fcs Ab pair, and their conversion from scFv-Fcs into IgGs, key steps in the generation of the ZEBOV D4 Assay.
5. Full-length antibody generation and characterization

The work presented thus far was aimed at generating Abs that will be integrated into the D4 assay for ZEBOV sGP detection. Before integrating these Abs into the assay, we sought to establish that they did not cross-react with Marburg virus, a pathogen in the same EBOV family. Cross-reactivity studies were followed by conversion of the scFv-Fcs into IgGs and characterization of full-length Ab binding to sGP from all relevant EBOVs. This work was performed to guarantee optimal Ab sensitivity and to ensure that the full-length Abs would have the capability of diagnosing not only ZEBOV, but also SUDV and BDBV, which have also caused recent outbreaks.

5.1 Cross-reactivity studies

We aimed to confirm that A1F3-1 and C2bA5-2 scFv-Fcs did not cross-react with Marburg virus (MARV) GP1 (MarGP). Ebola and Marburg viruses are both filamentous filoviruses and have similar clinical manifestations[153]. Marburg virus presents a glycoprotein that has ~30% homology with ZEBOV GP1 and sGP, a moderate level of homology, making testing of cross-reactivity necessary. Figure 33 shows results for Ag-down ELISA of MarGP with A1F3-1 and C2bA5-2, which demonstrate no cross reactivity with either Ab.

We also assessed Ab cross-reactivity towards GP. It was expected that these Abs would react to GP since sGP is a truncated version of GP containing most of its sequence. Figure 33 shows that both A1F3-1 and C2bA5-2 bind GP and sGP similarly. This reactivity
towards GP is welcomed as it provides a safeguard to reduce the probability of false-negatives in diagnoses during later stages of the infection, when sGP levels are reduced but GP levels are high.

![Figure 33](image.png)

**Figure 33:** A1F3-1 and C2bA5-2 Abs binding profiles to ZEBOV sGP, EBOV GP1, and MARV GP1, indicating lack of cross-reactivity of selected antibodies to MARV.

### 5.3 Conversion of scFv-Fcs into IgGs

We hypothesized that maximum sensitivity of the final assay would be achieved by using full-length Abs rather than scFv-Fcs, which lack the constant light chain (CL) and heavy chain (HC). Therefore we converted the scFv-Fcs into IgGs by subcloning the variable regions from A1F3-1 and C2bA5-2 into mouse gamma 2a and kappa chain expression vectors. Figure 34A depicts the strategy to generate the IgGs, which involved cloning DNA containing the Ab HC and LC into PCDNA5 using Gibson assembly[141]. This DNA was transfected into Expi293 cells for expression of equimolar amounts of heavy and light chain. We harvested expression supernatant and purified the IgGs using
the chromatography-free IsoTag-based purification strategy detailed in Figure 26 and section 3.3.2.2.

The outcome of each step of the purification process for A1F3-1 and C2bA5-2 can be seen in the SDS-PAGE gel in Figure 34B, which shows an effective Ab purification process. Figure 34C shows another SDS-PAGE gel with the final purification product for both Abs.

The IsoTag system to isolate the Abs is advantageous because it facilitates scale-up of the expression and purification process. Unlike column-based systems in which the amount of Ab that can be simultaneously purified is limited by the column bed volume, the IsoTag process can be scaled up by simply adding more ELP-ZD to the cell expression media.
Figure 34: Expression and purification of A1F3-1 and C2bA5-2 Abs. A) pcDNA5 expression vector design with cytomegalovirus promoter, azurocidin (AZ) signal peptide, and NheI and Xhol cut sites for monoclonal antibody heavy and light chain insertion. B) Isolation of A1F3-1 monoclonal Ab using IsoTag purification, run on an SDS-PAGE gel. Lanes 1-6: (1) cell culture extract before addition of IsoTag, (2) cell culture extract after addition of IsoTag, (3) cold-spin supernatant, (4) room temperature spin supernatant, (5) resuspended pellet following an extra cold-spin, (6) purified monoclonal antibody run under reducing conditions. C) Purified A1F3-1 and C2bA5-2 Abs on an SDS-PAGE gel. Lanes 1 and 2 for each construct show purified antibodies under (1) reducing and (2) non-reducing conditions.
5.3 Binding affinity towards other EBOV sGPs

Following the full-length Ab generation, we sought to characterize their binding affinity towards sGPs from 5 different EBOVs. This was done to determine the KDs of our selected Abs to sGP from other EBOVs besides the Zaire strain. This information could help us determine the application of these reagents towards generating a PAN EBOV diagnostic test. Besides this, we saw this as an opportunity to also demonstrate that full length Abs present better affinity than scFvs, which may translate in a diagnostic test with higher sensitivity.

Following a similar procedure as the one detailed in section 3.3.3, we employed a Protein A SPR chip and a standard Biacore T200 to determine the binding constants. To this end, we fitted the resulting sensorgram curves with the appropriate model. The sensorgram for each Ab with multiple sGP concentrations from ZEBOV, SUDV, BDBV, TAFV, and RESTV can be seen in Figure 35 and the average binding constants of three independent runs for each Ab are summarized in Table 5.

These results indicate that IgG2a full-length Abs present a 3-5 fold higher affinity towards sGP from ZEBOV. Importantly, SPR also demonstrated that A1F3-1 and C2bA5-2 also have the potential to be used to diagnose infection from SUDV, BDBV and RESTV, although A1F3-1 did not bind to sGP from TAFV and was less efficient at binding sGP from RESTV.
Figure 35: Binding constant determination of A1F3-1 (capture antibody) and C2bA5-2 (detection antibody) with multiple sGPs. Sensorgram of A1F3-1 and C2bA5-2 binding to sGP from ZEBOV (A-B), SUDV (C-D), BDBV (E-F), and RESTV (G-H) ebolaviruses. Only C2bA5-2 binding constant to sGP from TAFV (I) was determined, as A1F3-1 does not bind to the sGP from this ebolavirus. IgGs were bound to Protein A chips and different concentrations of sGPs were injected. Binding constants were determined using global fits and local Rmax of three independent sensorgrams based on the Langmuir (1:1) reaction model. KD = equilibrium dissociation constant.

Table 5: Binding constants for A1F3-1 and C2bA5-2 and multiple sGPs. Each data point represents the average and standard error of three independent
measurements. Kon = association constant; Koff = dissociation constant; KD = equilibrium dissociation constant.

<table>
<thead>
<tr>
<th>Mouse IgG2a Constructs</th>
<th>Binding Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>sGP Construct</td>
<td>Kon</td>
</tr>
<tr>
<td>Zaire</td>
<td></td>
</tr>
<tr>
<td>A1F3-1</td>
<td>4.91 ± 0.012 x 10^5</td>
</tr>
<tr>
<td>C2bA5-2</td>
<td>8.66 ± 0.647 x 10^5</td>
</tr>
<tr>
<td>Sudan</td>
<td></td>
</tr>
<tr>
<td>A1F3-1</td>
<td>1.42 ± 0.318 x 10^5</td>
</tr>
<tr>
<td>C2bA5-2</td>
<td>0.99 ± 0.246 x 10^4</td>
</tr>
<tr>
<td>Bundibugyo</td>
<td></td>
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<td>A1F3-1</td>
<td>2.37 ± 0.053 x 10^5</td>
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<tr>
<td>C2bA5-2</td>
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<tr>
<td>Reston</td>
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<td>7.09 ± 0.049 x 10^4</td>
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<tr>
<td>C2bA5-2</td>
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<td>Tai Forest</td>
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</tr>
<tr>
<td>A1F3-1</td>
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<tr>
<td>C2bA5-2</td>
<td>3.63 ± 0.308 x 10^3</td>
</tr>
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</table>

5.4 Methods

5.4.1 sGP expression and purification

gBlocks (Integrated DNA Technologies) containing SUDV, BDBV, RESTV, and TAFV sGP with a terminal HA tag, (genebank YP_138524, YP_003815436.1, AAV48578.1, AAB37092.1) were cloned in pDISPLAY vector (PMID: 15681442) using Gibson assembly.

*E. coli* containing the plasmids for each sGP were cultured overnight in Luria broth with ampicillin at 50 μg/mL. Plasmid DNA was purified using a Plasmid Plus Midi Kit (Genesee; USA). Purified DNA was Sanger sequenced (Eton Bioscience) and transfected into Expi 293 cells (Thermo Fisher) using Expifectamine. sGP proteins were purified from
culture supernatants using anti-HA-tag affinity chromatography and stored at -80°C in 1x PBS.

5.4.2 ELISA-based cross-reactivity assessment

Ag-down ELISA measurements were performed as previously described[137]. Briefly, ELISA assays were performed in Corning Costar high-binding immunoassay plates. All wash steps used 300 μL of 0.1% (v/v) Tween-20 in phosphate buffered saline (PBST) with 2 μg/mL glycoprotein (ZEBOV GP1 or Marburg GP1; Creative Diagnostics) or ZEBOV sGP in PBS by incubating the plate for 16 h at 4°C. After washing, the wells were blocked using 200 μL of blocking solution (2% w/v BSA, 5% v/v goat serum in PBS) for 2 h at room temperature. After washing, 100 μL of serial dilutions of purified Ab were added. After 1.5 h at room temperature, the plates were washed and 100 μL of peroxidase-conjugated goat anti-mouse IgG Fc antibody (Pierce) diluted 1:5000 in PBST was added. After 1 h at room temperature, the wells were washed and 100 μL of TMB substrate (3,3',5,5'-tetramethylbenzidine; Pierce) was added. After 15 min at room temperature, the reaction was stopped by adding 100 μL of 2 M sulfuric acid. Absorbance values at 450 nm were measured using a Molecular Devices Spectramax M2 plate reader.

5.4.3 Expression and purification of full-length antibodies

5.4.3.1. Generation of A1F3-1 and C2bA5-2 as IgG constructs

gBlocks containing variable light and mouse kappa chains for A1F3-1 and C2bA5-2 with appropriate overhangs were inserted into a pcDNA5 vector using Gibson assembly
(Gibson Assembly Master Mix, New England Biolabs). Using the same procedure, gBlocks containing variable heavy and mouse gamma-2a immunoglobulin sequences for A1F3-1 and C2bA5-2 were also inserted into a pcDNA5 vector through Gibson assembly. The resulting constructs were transformed into NEB 5-alpha competent cells (New England Biolabs) which were recovered with SOC media for 60 min at 37°C and later selected in TB agar plates with 50 μg/mL ampicillin. *E. coli* containing pcDNA5 vectors with inserts of interest were grown and isolated as previously described. Each plasmid was Sanger sequenced with appropriate primers to confirm the presence of the inserts.

**5.4.3.2. Expression of full-length Abs in mammalian cells**

IgG antibody expression was performed using an Expi293 high efficiency transient system. Isolated, sequenced plasmids for each light and heavy chain of A1F3-1 and C2bA5-2 were retransformed into NEB 5-alpha competent cells, plated, and cultured overnight on ampicillin-rich TB agar plates. Isolated colonies were grown in ampicillin-rich TB media, and DNA plasmid was purified using a QIAprep Spin Midiprep Plus Kit. Purified DNA was Sanger sequenced to guarantee correct expression products, and transfected into Expi293 cells. IgG plasmids with light and heavy chain were co-transfected at 1:1.5 heavy to light chain molar ratio at 1 μg of DNA/mL of culture. Culture supernatant was harvested 7 days after induction for Ab purification.
5.4.3.3. IsoTag-based IgG purification

This process was detailed in section 3.4.6. Briefly, 3.5 μM of IsoTag (Isolere Bio) was added per mL of culture, and the medium was centrifuged at 4°C followed by addition of salt to trigger phase transition, room temperature centrifugation, removal of supernatant, and resuspension of the resulting pellet. Following a final round of thermal cycling, the pellet was resuspended in pH 4 buffer and a final round of thermal cycling was formed. The resulting Ab-rich supernatant was separated from IsoTag pellet and neutralized using 0.1 mL of 1 M Tris pH 8.5 per mL of supernatant. Isolated Abs were buffer-exchanged with 10K Amicon centrifugal devices and concentrated to 1 mg/mL in 1x PBS.

5.4.4 Binding kinetics of IgGs to sGPs

Binding kinetics were determined by SPR using a Biacore T200 system (GE Healthcare). IgG solutions at 1 μg/mL in HBS-EP running buffer were injected in a protein A sensor chip at 5 μL/min until a final RU of ~350. For A1F3-1 and C2bA5-2 IgGs, different concentrations of ZEBOV, SUDV, BDBV, RESTV, and TAFV sGPs were injected at 30 μL/min for 200 s and dissociation was monitored for 600 s. Following each round of injection of IgG and sGP, the chip surface was regenerated using two 30 s injections of 10 mM glycine-HCl buffer pH 2 at 30 μL/min. Experimental data for Ab/Ag interaction were fit with a 1:1 binding model using global fits and local Rmax with BIA evaluation software.
(GE Healthcare). The A1F3-1 and RESTV sGP sensorgram was fit using a two-state reaction model with global fits.

5.5 Conclusions

Before conversion of the scFv-Fcs to IgGs, we determined whether the A1F3-1 and C2bA5-2 scFv-Fc Abs would cross-react with MARV GP. This was important as MARV also causes a hemorrhagic fever with similar symptoms as EBOV, and is endemic in some of the same regions of Africa as EBOV. This evaluation demonstrated no significant cross-reactivity. We next converted A1F3-1 and C2bA5-2 scFv-Fcs into IgGs using the same workflow previously validated. We rapidly cloned, expressed, and purified the Abs in significant amounts, aided by the chromatography-free IsoTag Ab purification process, which allowed us to process high volumes of material for both Abs simultaneously, without the need for extra equipment, simplifying the fabrication of these reagents.

Finally, we determined the binding affinities of these reagents to sGP from five major EBOV subtypes. These studies confirmed that full-length Ab versions of A1F3-1 and C2bA5-2 have higher affinity for these clinically relevant sGPs than their scFv-Fc counterparts, and showed that they are exceptional candidates for generation of an immunoassay that targets EBOV sGP. The next chapters will detail the ZEBOV D4 assay test development, its analytical validation, and studies with non-human primates.
6. D4 assay development

Our work up to this point was focused on generating optimal Abs for integration in the D4 assay, as part of our overarching objective of generating an assay for early detection of EBOV infection. Throughout the Ab generation process, we identified and addressed several key elements in the IVD test development workflow that were bottlenecks for reagent generation. These advances culminated in the rapid isolation of a high affinity Ab pair targeting non-overlapping epitopes of ZEBOV sGP. Employing these Ab reagents, we now present the development and analytical validation of an ultra-sensitive ZEBOV D4 POCT.

6.1 Integration of A1F3-1 and C2bA5-2 IgG in D4 assay

As previously described in chapters 1 and 2 and depicted in Figure 36A, we inkjet-printed cAb (A1F3-1) in PBS as spots in the central region of the chip, and FL-dAb (C2bA5-2) in PBS with 10% trehalose as “soluble” detection spots around the cAb spots. Following a brief drying step, we evaluated assay performance using ZEBOV sGP spiked in FBS. The dose-response curve (Figure 36B) indicated a LoD of 0.02 ng/mL. For reference, FDA-approved LFAs targeting other EBOV proteins have LoDs >10 ng/mL. In addition, these traditional viral targets are produced in lower amounts than sGP, and remain intracellular until there is active viremia and contagion (detailed in section 3.2 and in[124-126, 131, 134-135]). The low LoD for the D4 assay for ZEBOV sGP suggests that the D4 assay may have higher clinical sensitivity than the current gold-standard POCT platform.
Figure 36. Integration of cAb (A1F3-1) and dAb (c2bA5-2) in the D4 assay. A) Fabrication strategy in which capture and detection reagents are printed on a POEGMA-coated glass surface. B) Dose-response generated using sGP-spiked FBS, showing a LoD of 0.02 ng/mL.

We next evaluated the performance of the assay in human serum, to assess potential cross-reactivity with the abundant immunoglobulins and other components in human serum samples. We repeated the fabrication process and determined the assay’s LoD using sGP-spiked pooled human serum samples. The results (Figure 37) showed a troubling 300-fold loss in sensitivity, caused by a significant increase in the background of the capture spots even in samples with no sGP added, indicating cross-reactivity. We next sought alternatives to mitigate this problem.
6.2 Assay optimization

6.2.1 Mitigation of cross-reactivity with human samples

Sandwich immunoassays fabricated with monoclonal Abs are susceptible to cross-reactivity in human serum samples caused by human anti-mouse (HAMA) Abs[154-155]. These HAMA Abs, which are present in up to 50% of the population, can cross-link cAb and dAb and cause false-positive results.

To circumvent this issue, we were inspired by EBOV's strategy of delivering high amounts of a decoy protein (sGP) which depletes the immune system of GP-targeting elements. To enable this strategy, we integrated a copious amount of non-reactive mouse Abs which were mixed into the FL-dAb solution. We hypothesized that these Abs would be preferably targeted by HAMA Abs versus FL-dAbs and cAbs. To this end, we
employed a commercially available reagent, TruBlock, which reduced the assay background and re-established the sub-ng/mL LoD observed when using sGP spiked into FBS (Figure 38).

![Dose-response curve generated using sGP-spiked pooled human serum in a ZEBOV D4 assay with integrated TruBlock, showing a LoD of 0.02 ng/mL.](image)

**Figure 38:** Dose-response curve generated using sGP-spiked pooled human serum in a ZEBOV D4 assay with integrated TruBlock, showing a LoD of 0.02 ng/mL.

### 6.2.2 Redesigning the D4 assay for increased fabrication throughput

However, the addition of large amounts of non-reactive IgGs to the dAb printing solution presented a new set of challenges regarding assay fabrication. The increase in solution viscosity rendered the piezoarray-based printing of detection reagents useless. Film formation on the glass capillary tips and lack of consistent drop ejection resulted in constant fabrication run failures. Therefore, we redesigned the D4 assay, formerly composed of three rings of dAb printed around the cAb, to a single ring composed of larger spots printed using a non-contact nanoliter dispenser using BioJet technology. This approach, depicted in Figure 39, reduced the number of arm movements by the printer,
resulting in a fabrication throughput of up to 5000 tests per day in a laboratory setting, a development that could address the current problem of low device availability in the field[156]. Furthermore, the pressure-based system, which relies on a fast-acting solenoid valve, can process viscous solutions with greater consistency than piezo-based platforms.

**Figure 39:** Fabrication workflow for original and redesigned D4 assays. Piezo and BioJet non-contact dispensing systems are respectively used to print detection spots. Capture reagent is printed with a Piezo-based system.
6.3 Analytical performance of ZEBOV D4 assay

6.3.1 A D4 assay for detection of multiple ebolaviruses

Employing this new fabrication strategy, we demonstrated that the D4 assay could detect sGPs from other EBOV subtypes, corroborating the SPR experiments (section 5.4.4), and confirming that A1F3-1 and C2bA5-2 Abs could be used to diagnose other clinically relevant EBOVs. We evaluated chips using sGPs from ZEBOV, SUDV, BUDV, RESTV, and TAFV in FBS and a 90 min incubation (Figure 40). Our assay achieved pg/mL sensitivity for all sGPs except for TAFV, for which no dose-response was observed, consistent with the Ag-down ELISA (inset in Figure 40) and SPR (Figure 35) results. The dose-response curves showed a 10-fold loss in sensitivity when detecting RESTV sGP, likely due to the lower affinity of A1F3-1 for RESTV sGP, which was observed in Ag-down ELISA experiments. These results demonstrate that the test device could diagnose other clinically relevant forms of EBOV (SUDV and BDBV) with similar sensitivity as for ZEBOV.
Figure 40: sGP detection with EBOV D4 assay. (A) Bottom: Schematic of an EBOV D4 assay fabricated on POEGMA-coated glass. (i-iii) Images of 24 D4 assay chips fabricated on a POEGMA-coated borofloat glass slide, with laser-cut acrylic and adhesive creating individual sample wells. (i-ii) A D4 assay before being run. Trehalose pads followed by fluorescently-labelled dAb and Trublock are printed in a concentric pattern. The cAbs are printed in the center of the chip and are indicated by outlined circles. (iii) A D4 assay after being run. Following incubation with sample containing sGP, intense fluorescent capture spots (white) and detection spots (red) can be seen. (B-E) Dose-response curves in D4 assay fabricated with A1F3-1 (capture) and C2bA5 (detection) monoclonal Abs with ZEBOV sGP (B), Sudan EBOV sGP (C), Bundibugyo sGP (D), Reston sGP (E), and Tai Forrest sGP (F) in FBS. Insets: individual binding profile of each Ab to the respective sGP.

6.3.2 Sensitivity versus incubation time in complex matrices

To evaluate how assay performance could vary depending on the type of sample used, we generated dose-response curves with ZEBOV sGP spiked in FBS, pooled human serum (HS), rhesus macaque serum (MoS), and single donor human whole blood (WHB). We used rhesus macaque serum because one of our goals in the development of the ZEBOV
D4 assay is to track sGP values in animal models of EBOV disease. We also sought to determine assay performance as a function of the time a sample is incubated, to ensure that maximum sensitivity would be achieved.

Figure 41: D4 assay performance in different samples with and without TruBlock. D4 assay dose-response curves using A1F3-1 cAb and FL-dAb C2bA5-2 for different incubation times. Each data point represents the average and standard error of four independent D4 assays. Dose-responses in dashed lines were fit using a 5-parameter logistic fit and were used to calculate LoB, LoD, and DR for each incubation time. A) D4 assay conducted on analyte-spiked calf serum. Dose-response curves and LoDs for incubation times of 15, 30, 60, and 90 min. B-F) D4 assay conducted on (B) analyte-spiked pooled human serum, (C) analyte-spiked pooled human serum without blocking reagent (TruBlock) integrated on test devices, (D) analyte-spiked rhesus macaque serum, (E) analyte-spiked single donor human whole blood, and (F) analyte-spiked single donor human whole blood without blocking reagent (TruBlock) integrated on test devices. Dose-response curves and LoDs are shown for incubation times of 15, 60, and 90 min.
Figure 41 shows the dose-response curves obtained in these experiments, and the results are compiled in Table 6. The sensitivity varies with incubation time until 60 min of incubation. After 60 min, additional incubation does not improve the LoD or DR. If TruBlock is not integrated in the device, the LoB and consequently the LoD of the test is significantly compromised. The increase in assay background was less evident in the single donor whole human blood, perhaps due to a lower concentration of HAMA Abs.

Table 6: D4 assay results for different sample matrixes and incubation times. LoB = limit of blank, LoD = limit of detection, DR = dynamic range, NB = no blocking reagent.

<table>
<thead>
<tr>
<th>Incubation (minutes)</th>
<th>Calf Serum</th>
<th>Human Serum</th>
<th>Macaque Serum</th>
<th>Human Blood</th>
<th>Human Serum (NB)</th>
<th>Human Blood (NB)</th>
</tr>
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<tbody>
<tr>
<td>15</td>
<td>LoB (ng/mL)</td>
<td>LoD (ng/mL)</td>
<td>DR (log10)</td>
<td>LoB (ng/mL)</td>
<td>LoD (ng/mL)</td>
<td>DR (log10)</td>
</tr>
<tr>
<td>0.10 0.27 3.27</td>
<td>0.10 0.31 3.21</td>
<td>0.16 0.43 3.07</td>
<td>0.01 0.09 3.74</td>
<td>4.54 7.53 1.82</td>
<td>0.08 0.36 3.14</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.004 0.03 4.28</td>
<td>&lt;0.001 0.03 4.21</td>
<td>0.004 0.06 3.98</td>
<td>&lt;0.001 0.01 4.59</td>
<td>1.14 1.80 2.44</td>
<td>&lt;0.001 0.20 3.41</td>
</tr>
<tr>
<td>90</td>
<td>0.02 0.03 4.19</td>
<td>&lt;0.001 0.04 4.12</td>
<td>&lt;0.001 0.06 3.94</td>
<td>&lt;0.001 0.02 4.36</td>
<td>1.45 3.11 2.21</td>
<td>&lt;0.001 0.11 3.68</td>
</tr>
</tbody>
</table>

Based on these results, we conclude that there is little variation in dose-response behavior and LoD using different samples types. These results are consistent with previous results using POEGMA surfaces, first seen by Hucknall et al.[6, 52], but are not common in the field of IVD, as different sample matrices typically yield variable results. These results demonstrate the importance of the POEGMA brush coating in reducing sample matrix-driven assay variability, in addition to increasing the signal-to-noise.

6.3.3 Analyte concentration determination by fluorescent readout

We next sought to demonstrate that analyte concentration can be determined by using a test’s fluorescence readout. We performed a double-blinded spike and recovery
experiment in which a batch of ZEBOV D4 assays was fabricated, and a trained technician used those tests to generate a dose-response curve that was fit with a 5-parameter sigmoidal function. Next, a second researcher prepared blinded samples of human serum (HS) spiked with ZEBOV sGP. These samples were used in a ZEBOV D4 assay, and the fluorescence readout was used to determine the sGP concentration in the samples using the fitted curve.

![Graph showing spiked analyte recovery](image)

**Figure 42:** Double-blinded spiked analyte recovery experiment. sGP concentrations are determined by output fluorescence intensity in spiked pooled human serum.

Figure 42 shows a nearly perfect correlation ($R^2 = 0.994$) of spiked analyte concentration with sGP concentration determined using fluorescence output.

### 6.3.4 Evaluating the risk of hook effect

Extensive reports have shown elevated viremia levels in EBOV patients[157], especially in later stages of the disease. Elevated viremia correlates with high concentrations of circulating virus and viral proteins, which can cause the well-established hook or prozone effect. In the hook effect, an immunoassay yields a negative
result in a positive patient due to the overwhelming amount of target protein present. This phenomenon is caused by saturation of cAbs and dAbs with target analyte, in which case no sandwich can be formed by the cAb [158-159].

Figure 43: 8-log dose-response curve (0.01-100000 ng/mL) using analyte-spiked calf-serum and a 90 min incubation. LoD = limit-of-detection; DR = Dynamic Range; Useful Range = range in which a positive diagnosis can still be determined although analyte concentration cannot be determined.

To investigate the susceptibility of the ZEBOV D4 assay to the hook effect, we generated an 8-log dose-response curve using a maximum sGP concentration of 0.1 mg/mL. The assay showed a dynamic range of 5.1 log, and a useful range (the range in which a positive diagnosis can still be obtained by the fluorescence output levels) greater than 6.3 log (Figure 43). This wide performance range mitigates the risk of a hook effect producing false-negative results due to high levels of circulating sGP and GP in patients with high viremia.
6.3.5 Accelerated stability studies

To be deployed in at-need areas, the ZEBOV D4 assay devices must be stable for long periods of time and survive transportation without refrigeration or complex transport apparatus. To gauge the test’s ability to retain its sensitivity and broad dynamic range following long-distance shipments and storage in tropical areas, we performed accelerated stability studies by generating dose-response curves with chips that were stored in aluminum pouches with silica gel bags and exposed to 37°C for 30 and 60 days.

![Assay Accelerated Stability in Desiccated Pouch](image)

**Figure 44**: Storage stability of the ZEBOV D4 assay. Chips were stored in aluminum pouches with desiccant material (silica gel) at 37°C, and dose-response measurements were performed at 30 and 60 days.

Figure 44 compares dose-response curves of ZEBOV D4 assays exposed to ZEBOV sGP-spiked samples after 30 and 60 days of storage at 37°C. The data shows no significant difference in performance between chips at 0, 30, and 60 days, demonstrating the independence of cold-chain, a key element for far-forward test deployment.
These results corroborate findings by Hucknall et al. [6, 52] and Joh et al. [57], which demonstrated that assays fabricated on POEGMA brushes present exceptional stability. We speculate that this stability is caused by the high oligo(ethylene glycol) density of the polymer brush, which may provide a protective environment that stabilizes printed proteins from denaturing processes. Unlike the previous studies, we demonstrated that low-cost and widely available aluminum pouches containing silica can be used to store D4 assay for transportation, an approach more conducive to large-scale fabrication.

6.3.5 Fluorescence output stability

Another key element on the ZEBOV D4 assay that we investigated for the first time was the capability to reliably maintain fluorescence output following an assay run. We foresaw that field applications may entail scenarios in which handheld fluorescence readers may malfunction, run out of battery, or be damaged while trying to reach isolated communities. Being able to collect a sample and run the test at the point-of-need then image the results at a later time would be an invaluable asset for an IVD test, which PCR, plate-based immunoassays, and LFAs do not possess. A key challenge in outbreaks is transporting collected samples under refrigeration to testing centers, a scenario that ideally should be avoided even if the tests cannot be imaged immediately.

To determine the environmental parameters and time spans following a test run at which the D4 assay would still reliably yield a positive or negative diagnosis, we performed assay runs and immediately quantified the test results. Next, we stored the
chips at ambient conditions (~25°C and 50% humidity) for 10 days, then reimaged the tests and assessed their dose-response behavior and LoDs (Figure 45A-C). We used tests exposed to sGP spiked into MoS incubated for 15, 60, and 90 min. We evaluated chips at a 60 day time point at which we saw a small increase in assay background and loss in sensitivity (Figure 45D), which we speculate was caused by rehydration of the brushes and diffusion of fluorescent molecules in the vicinity of the spots.

To determine whether the fluorescence output would be stable at environmental conditions comparable to tropical regions, we incubated chips run in FBS and HS at 37°C at 50% humidity without a pouch, and at 100% humidity in a pouch with desiccant. To study the effect of high humidity and temperature, we removed a set of chips from a pouch following a few days of heat exposure and stored them at 100% humidity and 37°C (Figure 45E-G). These results demonstrated that 37°C incubation at low humidity for extended periods of time does not compromise the fluorescence output. High humidity does increase assay background and compromise readings, but this can be circumvented by storing the chips in a pouch with desiccant following a test run. These findings, presented in Figure 45 and summarized in Table 7, when taken together with those seen in Figure 44, demonstrate excellent assay stability under conditions likely to be encountered in the field.
Figure 45: Post-assay fluorescence output stability. (A-C) D4 assay dose-response curves determined using analyte-spiked rhesus monkey serum and incubation times of 15, 60, and 90 min following 10 days of environmental exposure (ambient temperature and humidity). (D) D4 assay dose-response curves determined using analyte-spiked calf serum and a 90 min incubation time following 10 and 60 days of environmental exposure. (E) D4 assay dose-response curves determined using analyte-spiked calf serum and a 90 min incubation following 15 and 30 days of exposure to 37°C stored in a sealed pouch with desiccant. (F) D4 assay dose-response curves determined using analyte-spiked calf serum and a 90 min incubation on day 0 (red) and following 15 days of exposure to 37°C stored in a sealed pouch with desiccant (black). The curve in green shows the assay incubated at 37°C and 100%
humidity for 12 days following its removal from the pouch with desiccant. (G) D4 assay dose-response curves using pooled human serum and a 90 min incubation on day 0 and after 10 days of exposure to 37°C under 50% humidity. The data was fit with a 5-parameter logistic fit (omitted for better visualization of the data), which was used to calculate LoDs.

Table 7: Post-assay fluorescence output stability. D4 assay figures of merit for fluorescence output stability experiments using (i) analyte-spiked rhesus macaque serum and incubation times of 15, 60, and 90 min for 0 and 10 days of environmental exposure (ambient and humidity); (ii) analyte-spiked calf serum and 90 min incubation for days 0, 10, and 60 of environmental exposure; (iii) analyte-spiked pooled human serum and 90 min incubation for days 0 and 10 of exposure to 37°C and 50% humidity; (iv) analyte-spiked calf serum and 90 min incubation for 0, 15, and 30 days of exposure to 37°C stored in a sealed pouch with desiccant; (v) analyte-spiked calf serum and 90 min incubation for days 0 and 15 of exposure to 37°C stored in a sealed pouch with desiccant followed by 12 days of exposure to 37°C and 100% humidity. FOM = figures of merit (LoB, LoD, DR); LoB = limit of blank; LoD = limit of detection; DR = dynamic range.

<table>
<thead>
<tr>
<th>D4 Assay Fluorescence Output Stability</th>
<th>i.) Macaque Serum (Ambient Conditions)</th>
<th>ii.) Calf Serum (Ambient Conditions)</th>
<th>iii.) Human Serum (37°C 50% Humid.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation (minutes)</td>
<td>LoB (ng/mL)</td>
<td>LoD (ng/mL)</td>
<td>DR (log10)</td>
</tr>
<tr>
<td>15</td>
<td>0.17</td>
<td>0.43</td>
<td>3.07</td>
</tr>
<tr>
<td>60</td>
<td>0.004</td>
<td>0.06</td>
<td>3.91</td>
</tr>
<tr>
<td>90</td>
<td>&lt;0.001</td>
<td>0.06</td>
<td>3.94</td>
</tr>
</tbody>
</table>

iv.) Calf Serum (Dessicant Pouch - 37°C) 90 minutes incubation

v.) Calf Serum (Dessicant Pouch - 37°C followed by high humidity exposure at 37°C) 90 minutes incubation

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 15 + 12ND</th>
</tr>
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<td>LoB (ng/mL)</td>
<td>LoD (ng/mL)</td>
<td>DR (log10)</td>
<td>LoB (ng/mL)</td>
<td>LoD (ng/mL)</td>
<td>DR (log10)</td>
</tr>
<tr>
<td>0.001</td>
<td>0.04</td>
<td>4.13</td>
<td>0.04</td>
<td>0.05</td>
<td>4.01</td>
</tr>
<tr>
<td>0.02</td>
<td>0.03</td>
<td>4.17</td>
<td>0.03</td>
<td>0.05</td>
<td>4.05</td>
</tr>
</tbody>
</table>

6.4 Direct comparison with LFA tests

Following performance and stability studies, we sought to compare the D4 assay platform with standard rapid device tests (RDTs). Hence, we explore the development and fabrication of LFAs using our selected A1F3-1 (cAb) and C2bA5-2 (dAb). We employed a well-established fabrication workflow, depicted in Figure 46[160], which involved the synthesis of 15 nm gold nanoparticles using a citrate reduction reaction...
(Figure 46B), followed by titration with dAb to establish the minimal amount of dAb needed to coat and stabilize the GNP probes (Figure 46D)[161]. Next, we coated the conjugate pads with this GNP-dAb solution, which were then frozen and lyophilized for 72 h (Figure 46E). In parallel, we used non-contact dispensing to add the A1F3-1 cAb in the test line region and goat anti-mouse Ab in the control line region of nitrocellulose membranes that were pre-assembled on PVC backing (Figure 46C). The impregnated nitrocellulose membranes were then left to dry under vacuum for 24 h. In a dry room (humidity < 30%), we assembled the master sheets composed of the nitrocellulose with absorbent, conjugate and sample pads, and red blood cell filters (to enable testing with whole blood) (Figure 46F). Under controlled humidity, the master sheets were cut into 4 mm strips, placed into LFA-appropriate plastic cassettes, and packaged into aluminum pouches with desiccant for long term storage. We evaluated the LFA devices with ZEBOV sGP-spiked HS, WHB, and MoS. Sensitivity improved with longer incubation times, up to an LoD of 6 ng/mL, which was achieved at 60 min. Longer incubations did not improve sensitivity.
Figure 46: EBOV sGP lateral flow assay fabrication. (A) Illustration of lateral flow assay with PVC backing, sample pad, red-blood cell filter, conjugate pad with gold nanoparticle conjugated detection antibody (GNP-dAB), (C2bA5-2), nitrocellulose membrane with capture antibody (A1F3-1) on test line region and goat anti-mouse Ab in control line region and cellulose based wicking pad. (B) Illustration of Citrate reduction based 15 nm gold nanoparticle synthesis. (C) Test and control line impregnation on nitrocellulose membrane with non-contact dispenser. (D) Gold nanoparticle titration process, to determine optimal dAb titers for GNP stabilization. (E) Illustration of conjugate membrane impregnation, where GNP-dAb are deposited and freeze-dried on fiberglass. (F) Master sheet assembly and strips cutting. Impregnated nitrocellulose membrane is assembled on PVC backing sheet followed...
by overlapping conjugate and sample pads. Finally, wicking pad is assembled and individual 4mm strips are cut with automated guillotine cutter. (G) Test assembly images were individually cut assay strips are placed in plastic cassette and placed in aluminum pouch with desiccant material. Product name and lot numbers are printed on pouch during the sealing process. (H) EBOV sGP LFA performance evaluation depicting assay runs with different concentrations of analyte spiked human serum. 31.25 ng/mL of sGP yields a faint test line while at 250 ng/mL the positive result is more evident. (I) Compiled performance of LFA assays in human blood, calf serum, human serum, and Rhesus monkey serum. Presence of test line was evaluated at 15, 60, and 90 min to determine LoD.

These results indicated that, when using the same Abs for test fabrication, the D4 assay outperforms the gold-standard POCT sensitivity by at least 200-fold. In addition, the LFAs using our Ab pair showed a sensitivity superior to that of FDA-approved devices[125, 127].

6.5 Fluorescence readout with a handheld detector

The ZEBOV D4 assay readout shown thus far required burdensome tabletop fluorescence scanners, which are poorly suited for use in low-resource settings. Previously, we addressed this problem by using a cell phone-based detector, which resulted in a 20-fold loss in sensitivity when compared to tabletop scanners[57]. This performance loss was unacceptable for early detection of EBOV.

This loss in sensitivity, coupled to the short life cycle of cell phones, motivated our group to combine the latest advances in microscopy, mobile computing, and rapid prototyping to generate a dedicated platform to image D4 chips, called the D4Scope, presented in Figure 47.
Figure 47: Handheld fluorescence detector, the D4Scope. (A) Real D4Scope device fabricated from a 3D printed body and off the shelf components with D4 assay fluorescence spots on the screen. (B) 3D design of D4Scope highlighting core components and overall architecture with computer on a chip integrated with touchscreen and USB camera. (C) D4Scope illumination scheme, depicting an oblique angle laser excitation format exciting the fluorescent microarrays on the POEGMA coated slide at a 45° angle, bandpass filter, and Raspberry Pi 4 processing unit.

The D4Scope platform is a low-cost, battery-powered fluorescence detector that images microarray spots with high sensitivity. The fluorescence elements in the D4Scope are set in an oblique angle laser excitation format, and contain a red laser with high optical coherence which excites the fluorophores in the D4 assay. The longer wavelength photons emitted by the FL-dAbs are filtered by a bandpass optical filter and are captured by a high-efficiency camera (Figure 47C).

To validate this platform, we ran ZEBOV D4 chips with sGP spiked in HS, and scanned the tests first with a high efficiency GenePix tabletop scanner (Figure 48D) and then with the D4Scope (Figure 48E). Figure 49A shows the dose-response acquired with the D4Scope, and the linear correlation between fluorescence output between both
scanners ($R^2 = 0.9992$). Assay performance with both scanners was roughly equivalent: the LoD using the GenePix was 0.07 ng/mL, and 0.10 ng/mL using the D4Scope.

Next, we evaluated the D4 assay in the Galveston National Laboratory, a BSL-4 facility. Figure 48F shows the dose-response curve generated using analyte-spiked rhesus monkey serum performed by local personnel following a 30 min training session, and scanned using a tabletop fluorescence scanner on site (SensoSpot). The same chips were then scanned using the D4Scope (Figure 48G). Figure 49B shows the dose-response acquired using the D4Scope, and the linear correlation between fluorescence output between the SensoSpot and D4Scope ($R^2 = 0.9795$). Assay performance with both scanners was similar, as the LoD was 0.11 ng/mL using the SensoSpot and 0.19 ng/mL using the D4Scope.

We also compared the GenePix and SensoSpot by comparing the dose-response curves generated from chips imaged in both detectors. As can be seen in Figure 48A-C, the dose-response curves obtain using both tabletop scanners were equivalent even though they rely on different schemes to image and quantify fluorescence output.

Finally, a double-blinded analyte recovery experiment was performed at Galveston in MoS, and sGP concentrations were determined based on the fluorescence output values with a $R^2 = 0.9877$ (Figure 49A). Although we witnessed a small loss in assay sensitivity, the ZEBOV D4 assay demonstrated exceptional performance even when performed by a user with little training, and after the sample was shipped between states.
These results confirmed that sGP concentration could be determined from the fluorescence from D4 chips to enable determination of sGP levels in infected patients.

Figure 48: GenePix, SensoSpot tabletop scanners, and D4Scope performance. (A-C) Dose-response curve in FBS with 90 min incubation acquired with gold-standard GenePix and SensoSpot tabletop fluorescence scanners. Inset depicts linear correlation of fluorescence readout (C). (D-E) Dose-response curve in HS with 90 min incubation acquired with GenePix tabletop fluorescence scanner and D4Scope handheld fluorescence scanner. (F-G) Dose-response curve in MoS with 90 min incubation acquired with SensoSpot tabletop fluorescence scanner and D4Scope handheld fluorescence scanner at Galveston National Labs (BSL-4) by a technician trained for 30 min.
Figure 49: D4Scope performance in the field setting. (A) Dose-response curve with ZEBOV sGP spiked in pooled human serum imaged with the D4Scope. Inset depicts correlation of fluorescence readouts from gold-standard GenePix scanner. (B) Dose-response curve with ZEBOV sGP spiked in rhesus monkey serum imaged with the D4Scope conducted by technician in Galveston National labs (BSL-4) following a 30 min training session. D4 Chips were shipped during the summer to simulate real world conditions. Inset depicts correlation of fluorescence readouts from SensoSpot tabletop fluorescence scanner also on site. C) Double-blinded spiked analyte recovery experiment in BSL-4 where sGP concentrations were determined by output fluorescence intensity in rhesus monkey serum.

6.6 Methods

6.6.1 D4 assay fabrication

D4 assay fabrication followed procedures described in sections 2.3.3 and 4.5.1 with minor changes. C2bA5-2 dAb was conjugated to Alexa Fluor 647 and diluted with a trehalose solution in 1x PBS to a final concentration of 0.25 mg/mL FL-dAb, 10% (w/v) trehalose, and 6 mg/mL TruBlock (if blocking reagent was added). A1F3-1 cAb had trehalose added to a final concentration of 0.05% (w/v) trehalose in 1x PBS and 1 mg/mL of cAb. cAbs spots were printed in the central area of the chip using a sciFLEXARRAY S11 spotter (Scienion). FL-dAb was non-contact dispensed in a concentric pattern around the cAb spots using a Biodot AD1520 printer. The chips received a plexiglass gasket to
separate all 24 tests in one glass slide. The gaskets were fabricated by laminating 300LSE (3M) adhesive on plexiglass and laser cut to fit the array. Following assembly, the tests were left to dry, protected from light in a vacuum chamber (~25 in. Hg. with desiccant for 1 h.

6.6.2 Dose-response curves with sGP from relevant EBOV

After fabrication, dose–response curves were generated by exposing D4 microarrays to FBS spiked with sGP from ZEBOV, SUDV, BDBV, RESTV, and TAFV at different concentrations for 90 min incubation. Each point of the curves represents the mean and standard error of four replicates. Following incubation, the chips were rinsed with 0.1% Tween-20 (v/v) in 1x PBS and spun dry with a C1303 slide spinner.

Arrays were imaged and quantified using an Axon Genepix 4400 (photomultiplier gain, 750; excitation power, 100). LoD was determined by using low-concentration samples (LCS) and the mathematical formula LoD = LoB + 1.645σLCS, where LoB is determined by measuring the mean fluorescence intensity (μ) and standard deviation (σ) from twelve blank samples as LoB = μblank + 1.645σblank. DR was determined as the range of concentration from the LoD to the greatest concentration that had a fluorescent signal greater than 3σ of the next lowest concentration in the dilution series. Data was fit using a five-parameter logistic curve using OriginPro 9.0.
6.6.3 Dose-response curves, relevant sample matrices and different incubations

Dose–response curves were generated by exposing D4 microarrays to FBS (Avantor), single donor whole human blood (Innovative Research), pooled human serum (Innovative Research), and rhesus monkey serum (Innovative Research) spiked with sGP at different concentrations for incubation times from 15 to 90 min. Each point of the curves represents the mean and standard error of four replicates. Following incubation, the chips were rinsed with 0.1% Tween-20 (v/v) in 1x PBS and spun dry with a C1303 slide spinner. Chips were imaged and data analyzed as described in section 6.6.2.

6.6.4 Accelerated and fluorescence output stability studies

Chips were fabricated as described in section 6.6.1. Following fabrication, the chips were packaged in heat-sealed aluminum pouches (EASE-Medtrend) with 5 g bags of silica (EASE-Medtrend). Following incubation at 37°C and 100% humidity, the chips were scanned and the results quantified as detailed in section 6.6.2.

Fluorescence stability studies were performed with chips run with analyte-spiked HS, MoS, or FBS. After being scanned and quantified, the chips were stored at 25°C and 100% humidity or inside a heat-sealed aluminum with a silica bag at 37°C and 100% humidity for a determined period of time, after which they were reanalyzed. One set of chips was stored at 37°C and 100% humidity for 10 days prior being rescanned.
6.6.5 Fabrication and performance of lateral flow test with A1F3-1 and C2bA5-2 Abs

All reagents were obtained from MilliporeSigma unless stated otherwise. To fabricate sGP-targeting LFA devices, gold nanoparticles (GnPs) were synthesized using a citrate reduction technique[162]. Briefly, 1% (w/v) gold (III) chloride trihydrate and 4% (w/v) trisodium citrate dihydrate solutions were prepared in DI water. 1000 mL of DI water with 20 mL of 1% gold (III) chloride stock solution was brought to a boil under reflux. Under vigorous agitation, 20 mL of the stock solution of trisodium citrate dihydrate was added. After 20 min under reflux, the heat was turned off and the bright orange-red solution was left to cool overnight and stored at 4°C. The newly synthesized GnPs were conjugated to dAb (C2bA5-2) through chemisorption via thiol derivatives. This conjugation was achieved by buffer exchanging dAbs into a pH 7.4, 20 mM HEPES HCl buffer and the concentration adjusted to 1 mg/mL with a 10kDa Amicon Ultra centrifugal device. 10 mL of 200 mM HEPES HCl buffer was added to 90 mL of GnP to adjust its pH. Next, a dAb dilution series was made using 20 mM HEPES and the 1 mg/mL Ab stock solution. To titrate the GnP conjugation, 100 μL of pH-adjusted GnP solution was added to 100 μL of each aliquot of the dilution series. Next, 100 μL of 2 M NaCl solution was added. The ratio of antibody to GnP solution that maintained its original color (red), indicated the optimal Ab amount to be conjugated. Using this ratio, 1 mL of dAb at 1 mg/mL was diluted to a final volume of 32 mL with 20 mM HEPES pH 7.4 and was mixed with the pH-adjusted GnP solution under vigorous stirring. Following conjugation, the
GnP-dAb solution was left under mild stirring for 10 min. Next, under mild agitation, 10 mL of BSA (20%) and sodium azide (1%) were added, followed by 10 mL of 10x PBS. This final conjugate solution was stored overnight at 4°C.

Following conjugation, GnP-dAb was lyophilized on fiber glass membranes. To this end, sucrose was added to a final 2% concentration and this conjugate solution was evenly distributed at 200 μL of GnP-dAb solution per cm² of membrane on fiberglass sheets (EASE-Medtrend) that had been washed with 0.01% (v/v) Tween-20 in DI water and air-dried overnight. The conjugate solution-infused membranes were flash-frozen in liquid nitrogen and lyophilized for 72 h with a Labconco Freeze Dryer (Labconco). Following lyophilization, samples were removed and immediately stored under vacuum to avoid moisture exposure.

FF80HP nitrocellulose membranes (GE Healthcare) were impregnated with an AD1520 non-contact dispenser (Biodot). Test lines received cAb (A1F3-1) at 1 mg/mL with 0.05% trehalose with a dispensing rate of 1 μL of solution per linear cm. Control lines received goat-anti mouse Abs (American Qualex Antibodies) at 0.5 mg/mL with 0.05% (w/v) trehalose at the same dispensing rate. Following impregnation, membranes were left to dry overnight under vacuum.

In a controlled humidity environment (<30%), impregnated nitrocellulose, MF1 RBC filtering membranes (GE Healthcare), wicking and sample pads (EASE-Medtrend), and 30 cm x 5 mm conjugate strips were assembled on an adhesive PVC backing (EASE-
Medtrend) and cut into 4 mm-wide test strips with an automated guillotine cutter (AutoKun). Test strips were placed into plastic cassettes (EASE-Medtrend) with sample ports and test and control line references. Plastic cassettes were placed into aluminum pouches with 0.5 g silica desiccant bags and heat-sealed for long term storage.

Performance validation was assessed by adding 25 μL aliquots of FBS, MoS, HS, and WHB with different concentrations of ZEBOV sGP to LFA test devices through the sample port. Three drops of a running buffer with 0.1% Tween20 in 1x PBS were added immediately. Following 15, 60, and 90 min incubations, test devices were read by two different users. Assay performance was determined by running three independent dose-response curves with analyte-spiked samples in a 2-fold dilution series with a starting concentration of 1000 ng/mL and a final concentration of ~2 ng/mL. We determined that samples at 31 ng/mL were weakly positive with a 15 min run time, and positive within 60 min. Eight ng/mL samples were negative within a run time of 15 min, but weakly positive within 60 and 90 min. 4 ng/mL samples were always negative independent of incubation time. To confirm the 6 ng/mL LOD, samples at this concentration were added to five test devices. All became weakly positive within 60 min of incubation.

6.6.6 D4Scope fabrication and performance

The D4Scope was designed and fabricated by Jason Liu and consists of a 3D-printed body with integrated imaging and processing elements. The handheld detector’s fluorescence elements were mounted in an oblique angle laser excitation format in a 3D-
printed body designed using Solidworks software and fabricated using a Lulzbot Taz 6 with 1.75 mm polylactic acid (PLA) filament (HATCHBOX). The red 185 mW, 635 nm laser diode (Sharp) with high optical coherence in the fluorophore’s excitation band (594-633 nm) is set at a 45° angle, and excites the Alexa Fluor 647 on the EBOV D4 assay, which is placed on a chip holder that attaches to the D4Scope handheld detector. The photons emitted by the fluorophore are filtered by a F01-676/37 25 mm bandpass optical filter (Shemrock) embedded in the camera’s field of view, and are captured by a high efficiency, USB-compatible AcA3088-57um CCD camera (Basler) with a MC 100x lens (Opto Engineering). To control the camera and process and visualize the acquired images, a Raspberry Pi 4 (Raspberry) system-on-board computer with a 3.5-inch TFT touchscreen (UCTRONICST) was integrated into the design. Custom software was written to control the camera’s exposure time, gain, and digital shift. Images with patient information, acquired by a user through the simple touch of a button which activates the laser and records the microspotted image, are stored in the device and uploaded to a dedicated cloud Mango/DB server. The software also previews the chip images while they are being loaded onto the device, ensuring proper use of the device by the user. The entire system is powered by a 10,000 mAh Power Bank (Omars) mounted on the D4Scope.

To assess detector performance, optimized chips were exposed to ZEBOV sGP spiked in HS for 90 min, rinsed, dried, and scanned with Axon Genepix at 750 gain and 100% power. The same chips were loaded onto the D4Scope and imaged with a 1 s
exposure, digital shift of 4, and 12 db gain. Fluorescence values were normalized using the formula $F_{\text{norm.}} = \frac{(F_m - F_{\text{blank}})}{F_{20\text{ng/mL}}} + \text{cste}$, where $F_{\text{norm.}} = \text{normalized fluorescence}$, $F_m = \text{measured fluorescence}$, $F_{\text{blank}} = \text{average fluorescence of blank samples}$, $F_{20\text{ng/mL}} = \text{fluorescence of 20 ng/mL}$, and $\text{cste} = 10$. A set of chips was also transported to University of Texas Medical Branch (UTMB) BSL-4 facility, where a technician was trained for ~30 min on how to run the EBOV D4 assay and operate the handheld detector. Following training, EBOV D4 chips were exposed to ZEBOV sGP spiked in MoS for 90 min, rinsed, dried, and scanned with the D4Scope and a SensoSpot fluorescence scanner with a 1 s exposure.

Compatibility between the tabletop scanners and D4Scope was ensured by scanning a set of EBOV D4 assays chips incubated with ZEBOV sGP in FBS for 90 min with the Genepix 4400 and SensoSpot fluorescence tabletop scanners. Dose-response curves were fit using a 5-parameter logistical fit, and the output correlation amongst tabletop scanners and between tabletop scanners and D4Scope was determined by linear fit. All fits and plots were performed using OriginPro 9.0.

### 6.7 Conclusions

In this chapter we presented the development of the ZEBOV D4 assay employing the previously generated and down-selected Abs. With the Ab pair integrated into the D4 assay platform, we performed extensive evaluations of assay performance using different types of samples. Our first challenge was to mitigate cross-reactivity with human samples.
spiked with sGP. This roadblock was circumvented by integrating high amounts of decoy Abs that absorbed HAMA Abs, reducing cAb and dAb cross-linking, and restoring sub-ng/mL sensitivity. Although effective, this approach hampered our fabrication process, which was then re-engineered to handle viscous solutions, and resulted in an increase in fabrication throughput with the use of non-contact dispensers that printed larger volumes of FL-dAb.

Utilizing this process, we fabricated several batches of ZEBOV D4 assay that we used to demonstrate that the test can also be used to detect sGP from clinically relevant SUDV and BDBV. Furthermore, we demonstrated exceptional test performance with multiple types of samples, and determined a minimal incubation time to ensure optimal sensitivity. We determined that the test is virtually immune to the hook or prozone effect. We also ensured that the test is stable under tropical and transport conditions, and can be imaged days after being run. After stability testing, we compared the D4 assay platform with the gold standard POCT, the LFA. We developed a LFA test with A1F3-1 (cAb) and C2bA5-2 (dAb) which demonstrated superior sensitivity to FDA-approved tests, but was over 200-fold less sensitive than the ZEBOV D4 assay.

Finally, we imaged the D4 assay results using a low-cost, battery-powered handheld scanner with similar sensitivity as costly and bulky tabletop scanners, thus addressing the longstanding issue of delivering quantifiable results at the point-of-need with high sensitivity. The results presented in this chapter demonstrate the exceptional
performance the D4 assay and provide a framework for IVD test validation for future projects.
7. Nonhuman primate studies

Following development and validation of the ZEBOV D4 assay with analyte-spiked samples and demonstration of sub-ng/mL sensitivity, we sought to demonstrate that our test could rival qRT-PCR for disease detection. To this end, we started a collaboration with the laboratory of Dr. Thomas Geisbert at the University of Texas Medical Branch (UTMB), which is equipped with a BSL-4 containment laboratory. Dr Geisbert is well-known for generating EBOV animal models and for development and validation of vaccines, treatments, and diagnostic tests for filoviruses.

Our overarching goal with using animals challenged with EBOV was to demonstrate for the first time a time-course for sGP levels in circulation, and to correlate it with viremia. Moreover, we aimed to perform a controlled comparison with the D4 assay and gold standard qRT-PCR for viral detection.

7.1 Choice of nonhuman primate animal model

The choice of nonhuman primates (NHPs) as animal models for EBOV disease versus rodent models was based on extensive work by Dr. Geisbert and others which demonstrates that mouse models do not accurately reflect human disease as the viruses have to adapt in mice[163-166]. While ferret models using non-adapted viruses are available [167-168], they do not display the coagulation defects seen in humans and NHPs[169]. NHPs hence are the preferred animal for EBOV disease models as they accurately mimic the human infection[170-171]. Among NHPs, cynomolgus and rhesus
monkeys are susceptible to disease and lethality when exposed to any of the clinically important EBOVs (ZEBOV, SUDV and BDBV), and their symptoms are consistent with human infection[129].

7.2 Detecting ZEBOV infection with a D4 assay

Following ZEBOV D4 assay development, we validated the capabilities of our testing platform to perform early detection of infection using non-human primates challenged through an intramuscular (IM) injection. We chose a 1,000 plaque forming unit (PFU) IM challenge model to perform these studies, which is a standard EBOV infection model that allows direct comparison with other testing techniques and devices, and has been widely adopted in IVD validation procedures[128-129].

To assess the sensitivity of the ZEBOV D4 assay, low passage ZEBOV (Makona) was used to IM challenge 10 rhesus macaques with a 1000 PFU dose. Prior to infection, baseline serum samples were taken from each animal (Day 0). Given the need to fully anesthetize the animals to obtain serum samples, and a strict constraint on blood volume drawn, it was only feasible to obtain three samples from each animal during the infection course.

Sample time points were staggered to cover the time course of 6 days as depicted in Figure 50A. All samples collected had infectious virus quantified by plaque assay on Vero Cells, viral genome copy determined by qRT-PCR, and fluorescence levels from
ZEBOV D4 assays determined from images acquired with the D4Scope and the SensoSpot tabletop scanner.

These results, shown in Figure 50B-C and compiled in , show that baseline samples were negative by all assays. One sample was positive on Day 1 by ZEBOV D4 assay, based on fluorescence values higher than the D4 LoD and negative for all other assays. The four samples from Day 2 were negative by PCR, but three were positive by ZEBOV D4, albeit with fluorescence levels only marginally above the assay’s LoD. One of these samples positive on the D4 test also had a PFU = 8.3 in the Vero Cell test. All samples collected on Day 3 had high fluorescence values in the ZEBOV D4 assay and were also PCR- and plaque assay-positive (P<0.0001) (Figure 50B-C).

Samples from all time points after Day 3 were positive in all assays . There was evidence of a hook effect in the later time points of the D4 assay, presumably due to high levels sGP and GP associated with viremia in end-stage disease, confirmed by elevated PFU and genome copies counts; this result was surprising considering the extensive validation we performed with the ZEBOV D4 assay and careful prozone effect evaluation at up to 0.1 mg/mL sGP. These results indicate that in later stages of the infection, sGP + GP levels may reach mg/mL concentrations.

qRT-PCR and infectious virus counts (PFU) are depicted in Figure 50C. qRT-PCR results are only positive on Day 3, at least one day after 75% of samples presented fluorescence levels above the ZEBOV D4 LoD. These results indicate that the ZEBOV D4
assay matches qRT-PCR performance with high statistical significance (P<0.0001), a feat never achieved by an RDT platform or any immunoassay.

Importantly, plaque assays on Vero cells provide a measure of assay sensitivity, which can be used to compare the performance of our test with that of other RDTs without the need for side-by-side testing, which requires precious EBOV-positive samples and test devices which are often not commercially available. Hence, we used published results for comparison. We analyzed WHO Emergency Use Assessment and FDA public reports for Oraquick Ebola and ReEBOV, which reported sensitivities of ~1.0x10⁶ PFU. In the current ZEBOV (Makona) IM model, this sensitivity translates to positive results only on Day 5 of infection. The DPP Ebola Antigen System reports a sensitivity of ~2.0x10⁵ PFU, which translates to marginally positive results on Day 4 following infection, but one of the two samples collected on Day 4 showed a PFU <1.0x10⁵, which translates into a false-negative at this time point[125, 127].

The sGP-targeting LFA showed an analytical sensitivity of ~1.0x10⁶ viral genome copies. This sensitivity was later deemed to be ~3.0x10⁴ in a nonhuman primate model, although with a limited number of samples and a variation greater than 500% in the optical density readout of the test for biological replicates[172]. In the current format, this test would be able to positively diagnose samples on Day 4, a performance that surpasses current WHO- and FDA-approved RDTs but still 2 days later than the ZEBOV D4 assay.
These results demonstrate that current RDTs lack the sensitivity to match qRT-PCR performance when infected patients present low viral loads[125, 127].

Following this comparison of the D4 assay’s sensitivity versus other RDTs, we sought to establish a time course of sGP levels in circulation throughout the infection. We determined sGP levels using the dose-response in Figure 49B, as this experiment used chips from the same fabrication batch as employed in the animal studies. Measured sGP levels increased in a statistically significant manner with each subsequent day after infection (Figure 50D). We calculated a P<0.0001 for the log transform of the sGP levels from days 0–2 to days 3–6, rising from pg/mL levels to ng/mL by Day 3, and μg/mL by Day 4. Importantly, there was a linear correlation between calculated sGP and viral load determined by PCR (R² = 0.9921) for values in the D4 assay’s linear range (Figure 50E).

Finally, we validated the D4Scope results with the tabletop fluorescence scanner, which presented an R² = 0.9667 for the fluorescence output readout (Figure 50F).
Figure 50: sGP detection in non-human primates IM challenged. (A) Sample collection scheme for ten healthy filovirus-negative rhesus macaques IM challenged with 1000 PFU of ZEBOV Makona strain. Serum samples collected were tested in the ZEBOV D4 assay and an extensively validated qRT-PCR [173-174]. (B) Normalized fluorescence intensity levels acquired with the D4Scope are reported for each time point. Each fluorescence value is the average of three technical replicates. (C) Infectious virus quantified by plaque assay on Vero cells and viral genome copies were measured using qRT-PCR with primers targeting VP30. Statistically significant difference of the log transform of PCR and PFU/mL readouts starts on day 3 as determined by one-way ANOVA followed by Tukey’s post-hoc test (P<0.0001). Estimated results based on infectious particle counts (PFU/mL) for FDA and WHO approved VP40 targeting Oraquick Ebola Rapid Antigen Test and Corgenix ReEBOV Antigen rapid tests are depicted and compared with PCR and D4 assay test results. *DPP Ebola Antigen System indicate marginally positive results on day 4 according to reported sensitivity in FDA emergency use authorization. (D) sGP levels timeline was determined by converting fluorescence intensity to sGP concentration. Each data point is the average and standard deviation (S.D.) of sGP values of each animal. Day 2 indicates pg/mL levels of sGP (above the assays LoD). Statistically significant difference of the log transform of sGP concentration starts on day 3 as determined by one-way ANOVA followed by Tukey’s post-hoc test (P<0.0001). Means indicated by
the same letter are statistically significant. (E) Correlation between sGP concentration and viremia determined by qRT-PCR. pg/mL sGP levels are detected on day 1 on 25% of samples and on day 2 on 75% of samples tested where PCR indicates negative results. Inset depicts correlation of viral load and sGP concentration in the linear range of the D4 Assay. (F) Correlation between fluorescence output of D4Scope and SensoSpot tabletop fluorescence scanner. Each data point is the average and standard deviation (S.D.) of sGP values of each animal. qRT-PCR LoD = \( \sim 10^3 \) (RNA/mL). Positive or negative results for PCR and D4 are color-coded in the panels. LFA results were estimated based on infectious particle count results. PFU = plaque-forming units; UN = undetermined.

Table 8: qRT-PCR and PFU results (log10 of RNA copies per mL) in non-human primates IM challenged. Normalized D4 assay fluorescence levels in triplicate and sGP levels calculated using the dose-response from same fabrication batch. “-” = assay not performed; LOD = limit of detection.

<table>
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<th>Macaque ID</th>
<th>Log10 copies/mL</th>
<th>Log10 PFU/mL</th>
<th>Normalized Fluorescence</th>
<th>sGP Conc. (ng/mL)</th>
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<td>Day</td>
<td></td>
<td></td>
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<tr>
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7.3 A second model of ZEBOV infection

The results obtained by testing EBOV-infected nonhuman primates throughout several days following IM challenge indicated that the D4 assay could match the sensitivity of qRT-PCR, as 75% of samples tested on Day 2 were D4-positive and PCR-negative. However, the large spike and variability between sGP levels in monkeys from Day 3 meant that the positive results from Day 2 had no statistical significance.

To demonstrate that we could match or surpass PCR sensitivity, we tested a different set of nonhuman primates challenged through a different route of infection that yields a slower and more natural progression of the disease. To this end, we selected a conjunctival (CNJ) exposure infection model[175].

As previously done, low passage ZEBOV (Makona) was used to challenge six rhesus macaques with a 10,000 PFU dose applied to the medial canthus of the eye. Prior to infection, baseline serum samples were taken from each animal (Days -8 or 0). Unlike the IM model which rapidly evolves into severe disease, the CNJ model evolution is slower and allows for collection of more blood samples from each animal.

Sample time points were staggered to cover the time course of 10 days (Figure 51A). All samples collected had infectious virus quantified by viral genome copy determined by qRT-PCR and/or fluorescence levels from ZEBOV D4 assays determined from images acquired with the D4Scope and SensoSpot tabletop scanner.

These results, compiled in Figure 51B-C and in
Table 9, show that baseline samples were negative by all assays. One sample was strongly positive on Day 4 (all three technical replicated) by ZEBOV D4 assay and negative for qRT-PCR. Out of four samples from Day 5, two were PCR-negative and two weak positives. No D4 testing was done on these samples due to limitations in sample volume. Samples from all time points after Day 5 were positive in all assays. There was also evidence of a hook effect in samples collected at Day 10, which we believe was caused by high levels of sGP and GP associated with viremia in end-stage disease, confirmed by elevated genome copies counts. These results indicated that the ZEBOV D4 assay surpasses qRT-PCR sensitivity.

Similar to previous results presented for the IM model, we sought to establish a time course of sGP levels in circulation throughout the infection. We determined sGP levels with the dose-response in Figure 49B, as this experiment used chips from the same fabrication batch as employed in the animal studies. Measured sGP levels increased in a statistically significant manner with each subsequent day after infection (Figure 51D). We calculated a P=0.098 for the log transform of the sGP levels between baseline and Day 4, which indicates that sGP levels rise from sub-pg/mL levels to ng/mL by Day 4, and μg/mL by Day 7. Finally, we validated the D4Scope results with the tabletop fluorescence scanner which presented an $R^2 = 0.9731$ for the fluorescence output readout (Figure 51F).
Figure 51: sGP detection in non-human primates CNJ challenged. (A) Sample collection scheme for ten healthy filovirus-negative rhesus macaques CNJ challenged with 10000PFU of ZEBOV Makona strain. Serum samples collected were tested in the ZEBOV D4 assay and an extensively validated qRT-PCR [173-174]. (B) Normalized fluorescence intensity levels acquired with the D4Scope are reported for each time point. Each fluorescence value is the average of three technical replicates. (C) Infectious virus quantified by viral genome copies measured using qRT-PCR with primers targeting VP30. Statistically significant difference of the log transform of PCR on day 7 as determined by one-way ANOVA followed by Tukey’s post-hoc test (P<0.0001). (D) sGP levels timeline was determined by converting fluorescence intensity to sGP concentration. Each data point is the average and standard deviation (S.D.) of sGP values of each animal. Day 4 indicates ng/mL levels of sGP for one sample (above the assays LoD). Means indicated by the same letter are statistically significant. (E) Correlation between sGP concentration and viremia determined by qRT-PCR. (F) Correlation between fluorescence output of D4Scope and SensoSpot tabletop fluorescence scanner. Each data point is the average and standard deviation (S.D.) of sGP values of each animal. qRT-PCR LoD = ~10^3 (RNA/mL). Positive or negative results for PCR and D4 are color-coded in the panels. UN = undetermined.
Table 9: qRT-PCR results (log10 of RNA copies per mL) in non-human primates CNJ challenged. Normalized D4 assay fluorescence levels in triplicate and sGP levels calculated using the dose-response curve from same fabrication batch. “-” = assay not performed; LOD = limit of detection.

<table>
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<th>Macaque ID</th>
<th>Day</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; copies/mL</th>
<th>D4 Assay Normalized Fluorescence</th>
<th>sGP Conc. (ng/mL)</th>
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7.4 Methods

7.4.1 Virus and challenge

The samples from the IM model[176] used in this work and from the CNJ model have been previously collected and characterized in studies approved by the University of Texas Medical Branch (UTMB) at Galveston Institutional Animal Care and Use Committee (IACUC) and UTMB’s Institutional Biosafety Committee. Briefly, a seed stock of EBOV Makona from a 2014 fatal human case originated at Guékedou, Guinea was used. Following passage in authenticated Vero E6 cells (ATCC, CRL-1586), ZEBOV isolate H.sapiens-tc/GIN/2014/Makona-Guekedou-C07, accession number KJ660347.2 was produced. This isolate was used to challenge 10 healthy, filovirus-negative male cynomolgus macaques 3–5 years of age and between 4–8 kg with 1,000 PFU intramuscularly or 10,000 PFU conjunctivally. Animals were housed in the Biosafety Level 4 (BSL-4) laboratory in the Galveston National Laboratory (GNL) and monitored post challenge for clinical signs of disease.

7.4.2 Virus detection and quantification

Viral titer was determined by plaque assay on Vero E6 cells and RNA quantification by RT-PCR with probes targeting VP30 gene[128]. As reported elsewhere in greater detail[176], cells were plated and grown to confluency and virus was titrated in duplicate from $10^{-1}$ to $10^{-6}$ and counted with neutral red stain. RNA was isolated from whole blood (WB) using AVL Buffer and a Viral RNA Mini Kit (QIAGEN). Primers
targeting ZEBOV Makona were used in the RT-qPCR assay[174]. CFX96 (BioRad Laboratories) was used to detect EBOV RNA, employing a One-Step Probe qRT-PCR Kit (QIAGEN) with a cycle of 50°C for 10 min, 95°C for 10 s, and 40 cycles of 95°C for 10 s followed by 59°C for 30 s. Threshold cycle values representing ZEBOV genome equivalents (GEq) were analyzed with CFX Manager Software, and data is shown as mean ± SD of technical replicates. GEq standard was created from RNA from ZEBOV stocks that was extracted and the number of ZEBOV genomes calculated with Avogadro’s number and the molecular weight of the ZEBOV genome. Plaque assays presented an LOD of 25 PFU/mL[177] and RT-PCR 1000 genome copies/mL.

7.4.3 sGP detection in nonhuman primate models

Previously characterized samples from 10 Rhesus monkeys IM challenged and 6 Rhesus monkeys CNJ challenged were added to the ZEBOV D4 assay. Following ~90 min of incubation, chips were rinsed and imaged using the D4Scope and SensoSpot fluorescence scanners. Each data point of fluorescence level reported is the average of three technical replicates. sGP levels were calculated employing a dose-response from the same batch of tests.

7.4.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, Inc). One-way ANOVA followed by Tukey’s posthoc multiple comparisons test was used to evaluate the statistical significance of differences between groups.
7.5 Conclusions

The results presented in this chapter demonstrate for the first time that a sandwich immunoassay POCT can match the sensitivity of qRT-PCR for ZEBOV detection. Importantly, these results were obtained using a low-cost, battery-operated handheld detector, which can be easily deployed at the point-of-care.

Our initial studies employed a well-established IM model of the infection, in which the D4 assay matched qRT-PCR sensitivity (p<0.0001). Although we also obtained evidence that our assay could surpass PCR sensitivity with multiple PCR-negative samples testing positive one day before detectable viremia, these results were not statistically significant due to the elevated spike in sGP levels at Day 3 post challenge.

To demonstrate that our assay surpasses PCR sensitivity in detecting EBOV infection, we employed a second nonhuman primate model with a CNJ challenge route. This model presents a slower progression of the disease, which resulted in low ng/mL sGP (P = 0.098) levels on Day 4 following challenge, which is one day before 50% of samples collected tested PCR-negative (Day 5). Using the D4 assay, we also established the time course for sGP + GP1 levels throughout the infection.

These encouraging results are the culmination of a multi-laboratory, multi-investigator project which aimed to reimagine the process of IVD testing development, and how EBOV disease is diagnosed.
8. Conclusions

The field of in vitro diagnostics has seen a surge in interest due to wider use of highly sensitive and reliable PCR-based sensing techniques for viral detection, driven mainly by recent infectious disease outbreaks (Ebola, Dengue, and Zika). Another factor driving IVD research and development is a pivotal shift from the model of centralized laboratory-run assays to widely available platforms that can be used at the point of care to obtain rapid results and more frequent tracking of pathophysiological biomarkers, to achieve earlier diagnosis and better disease management.

This growing interest in IVD has resulted in efforts to miniaturize and simplify PCR-based testing workflows, but DNA and RNA sensing is still limited in its widespread use in low-resource settings. The gradual replacement of immunoassays for infectious disease testing by PCR has been driven by a lack of sensitivity, which prevents detection of antigens in early stages of infection. Efforts by our group to address this intrinsic lack of sensitivity resulted in the development of POEGMA-coated immunoassay surfaces that block background protein adsorption and yield femtomolar levels of sensitivity when used to fabricate sandwich immunoassays.

Following development of the D4 assay platform, our efforts were aimed at understanding the surface properties responsible for POEGMA’s protein resistance that also enabled simple immunoassay fabrication. This investigation culminated in a process to rationally engineer the required parameters within a different class of polymer brushes,
resulting in POEGMA-like assay performance. These results highlighted that POEGMA-coated surfaces present the physicochemical properties that are optimal for IVD test development and high-throughput device fabrication.

These findings steered us to focus on key elements of the immunoassay development workflow, namely the laborious and time-consuming process of generating antibodies. With expedited antibody generation, POEGMA-based immunoassays can rival or replace PCR as the method of choice to manage ongoing infections and contagion. We demonstrated that reagent generation for IVD tests can be expedited by coupling M13 phage display Ab development with transient protein expression, chromatography-free Ab purification, and D4-enabled Ab pairing. This new reagent development workflow was effective at generating high-quality Abs that target a new IVD biomarker in EBOV disease (sGP), which we speculated could yield diagnosis prior to onset of symptoms.

Following Ab generation, we demonstrated that a novel POCT platform, the D4 assay coupled with a handheld low-cost detector, can be used to generate IVD tests that are independent of the cold chain, can be mass-produced, and exhibit low LoDs, broad DRs, and exceptional performance independent of the type of biological sample used. Finally, we demonstrated the clinical relevance of such a test by detecting EBOV sGP in two distinct nonhuman primate models of infection, at least one day before PCR could detect the presence of the virus.
We believe these exceptional results could only be achieved by combining the novel ultra-sensitive D4 assay platform with targeting of a protein that is not part of the virion, but rather expressed and secreted as a decoy to subvert the immune system.

8.1 Future paths for immunoassay development

Although we addressed several key elements in immunoassay development, there are several challenges that will require inventive solutions, especially regarding antibody generation. Our reliance on immunizations and laborious screenings to generate Abs can add weeks or months to the test development process.

This concern has been partially mitigated by the use of novel expression vectors which allow a seamless transition between E. coli and mammalian expression systems, coupled to next generation sequencing to identify consensus sequences for the CDRs on V\textsubscript{L} and V\textsubscript{H} of Abs, which could streamline antibody generation. But even equipped with these new tools, Ab generation still requires animal immunizations that can last several weeks.

Methods for synthetically generating (non-Ab) binders with high affinity and specificity could obviate the need for immunizations. One candidate we foresee having widespread IVD applications is based on the fibronectin type III domain (Fn3), which presents an Ab-like scaffold with interconnecting loops that mimic the variable regions of Abs, and can be designed/evolved to bind antigen targets. The small size of Fn3 domains can be leveraged to engineer multivalent structures which can reach binding affinities that
are unattainable by traditional Abs (K<sub>D</sub> < 10<sup>12</sup>). These structures can also be designed to target multiple targets that are secreted or shed into circulation or are part of the viral capsid, enabling development of immunoassays with even greater sensitivity.

### 8.2 Missing elements in the D4 POCT

The cornerstone of this project was the use of POEGMA-coated D4 tests as a reagent screening, pairing, and testing platform. Unlike other testing platforms, the D4 assay expedites and simplifies device fabrication while enabling quantitative readout in a high-sensitivity test adequate for field use. Further development is required to address issues such as the assay’s open format, which increases the risk of exposure to healthcare workers, and the need for a final rinse step to remove unbound proteins and molecules on the surface of the test prior to fluorescence readout. Both of these issues can be addressed by integrating microfluidics chips that encase the D4 assay elements and control the incubation and washing steps, preferably without the need of active pumps, which increase complexity and cost. We are currently working on solutions to these challenges that will enable the translation of our D4 assay platform into a sensing tool widely used in the clinic.
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Biography

Cassio Mendes Fontes graduated from Electronics and Telecommunication Engineering from Pontificia Universidade Catolica de Minas Gerais and Pharmacy and Biochemistry from the Federal University of Minas Gerais, where he also earned the title of Masters in Electric Engineering. Cassio was awarded a Science Without Borders fellowship in the fall of 2014 and then joined the Chilkoti Laboratory in the Department of Biomedical Engineering at Duke University. While at Duke Cassio earned a Masters in Biomedical Engineering in the fall of 2018 and helped produce the following scientific publications:


