Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Functional comparison of paper-based immunoassays based on antibodies and engineered binding proteins

Ki-Joo Sung[1], Yara Jabbour Al Maalouf[1], Quinlan Johns[1], Eric Miller[1], Hadley D. Sikes[1,2,*]

[1]Department of Chemical Engineering, Massachusetts Institute of Technology,

Cambridge, Massachusetts 02139

[2]Singapore-MIT Alliance for Research and Technology Centre (SMART)

1 CREATE Way, INNOVATION Tower, Singapore 138602

*Corresponding Author:
Professor Hadley Sikes
Telephone: (617) 253-5224
Fax: (617) 253-5224
Email: sikes@mit.edu

Materials and Methods

Commercial reagents

Mouse anti-ZNS1 antibody ("mouseAb") was purchased from Abcam (Abcam ab218547, clone D11), rabbit anti-ZNS1 antibody ("rabbitAb") was purchased from GeneTex (GeneTex GTX133307), and biotinylated mouse anti-ZNS1 antibody ("bAb") was purchased from Arigo Biolaboratories (clone SQab1610). Streptavidin AF647 ("SA AF647"; S-21374) was sourced from Thermo Fisher Scientific. Sterile-filtered human serum was from human male AB plasma of USA origin (Sigma-Aldrich H4522).

Cloning of ProA-CBD gene construct

The CBD construct was sourced from a pET28b-rcSso7d-CBD plasmid developed previously.[1] The plasmid containing the original ProA2 construct is plasmid pKK-TEV-ProteinA (Plasmid #105788) and was purchased from Addgene. Polymerase chain reaction (PCR) amplification was conducted using the two primers listed in the Table S1. The annealing temperature used for the procedure was 57.5 °C. The samples were run on 1% agarose gel and then the amplified protein A was isolated using a DNA gel extraction kit. Afterwards, the isolated protein A and the pET28b-rcSso7d-CBD were both incubated with NdeI and BamHI restriction enzymes at 37 °C for one hour. The digested products were then run on 1% agarose gel and then isolated using a DNA gel extraction kit from Epoch Life Science**.** All ligation mixtures were purified using the DNA Clean and Concentrator-5 Kit from Zymo Research (Irvine, CA, USA), and eluted in 12 μL of PCR-grade water. The digested plasmid backbone and protein A product were then co-incubated at room temperature for 10 minutes with T4 DNA ligase and 10x ligase buffer. 4 μL of this ligation product was transformed into DH5α *E. coli* (Fφ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) gal- phoA supE44 λthi-1 gyrA96 relA1) via electroporation. The entirety of this transformation mixture was plated on LB-kan plates and incubated overnight at 37 °C. Positive clones were verified via both N- and C-terminal sequencing, using the T7 promoter and T7 terminator sequencing primers.

Table S1: Oligonucleotide sequences of primers used in the cloning of the ProA-CBD construct.

#	Oligo Name	DNA Sequence (NdeI, BamHI restriction sites)
1	ProA - forward	5'-GGCGCGCATATGGTGGACAACAAATTC -3'
2	ProA - reverse	5'-TATGTAGGATCCTTTCGGCGCCTGAGC-3'

Production of recombinant proteins

Plasmids for SsoZNS1.E2-CBD and b-MBP-SsoZNS1.E1 were cloned previously, and they were expressed and purified as previously described.[2] Recombinant ZIKV NS1 biomarker (ZNS1) and DENV2 NS1 biomarker (D2NS1) were expressed and purified as previously described.[3] Plasmids for ZNS1 and D2NS1 biomarkers were sourced from Sino Biological. All recombinant proteins were produced in BL21(DE3) *E. coli* and induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The b-MBP-SsoZNS1.E1 variant (containing an N-terminal biotin acceptor sequence AviTag: MAGGLNDIFEAQKIEWHE) was supplemented with 0.1 mM D-biotin (97061-444, VWR) during expression. After overnight expression at 20 °C, the cells were lysed via sonication. The clarified lysate was used for purification for all recombinant proteins except ZNS1 and D2NS1. For ZNS1 and D2NS1, since the recombinant protein was expressed in the insoluble fraction, denaturing buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 8 M urea, pH 7.6 at room temperature) was added to the insoluble lysate pellet and incubated overnight at 4 °C to solubilize the protein. The resulting supernatant was used for purification.

Since all recombinant proteins contain an N-terminal 6x-histidine tag, the protein products were purified using immobilized metal affinity chromatography (IMAC) using HisTrap FF crude columns (GE Healthcare). rcSso7d-based proteins were buffer exchanged into 40 mM sodium acetate (pH 5.5) using Amicon Ultra Centrifugal Filters (10K MWCO). ProA-CBD was buffer exchanged into 1x PBS (phosphate buffered saline) using Amicon Ultra Centrifugal Filters (10K MWCO). The NS1 proteins were refolded with slow dialysis (50 mM Tris, 300 mM NaCl, 400 mM L-arginine, 1 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized), pH 7.6 at room temperature) using Slide-A-Lyzer dialysis cassettes (10K MWCO, 12 mL, ThermoFisher

Scientific) after diluting the NS1 proteins below 100 μg/mL prior to dialysis to minimize aggregation.

All purified proteins were quantified using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). To verify purity, the proteins were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) with Biorad P/N 161-0374 used as the protein ladder. The gel was stained with Coomassie Brilliant Blue G-250.

After IMAC purification and buffer exchange, b-MBP-SsoZNS1.E1 was further purified on a Pierce Monomeric Avidin Agarose Kit (Thermo Fisher Scientific) to purify the proteins with accessible biotins. The kit protocol was followed, and the provided Regeneration buffer was used for elution. The elution fractions were pooled together and concentrated down using Amicon Ultra Centrifugal Filters.

Fabrication and testing on cellulose assay test strips

Whatman No. 1 chromatography paper was oxidized for aldehyde functionalization as described previously.[4] Hydrophilic test zones were printed on non-functionalized paper (for CBD-based protein assays) and oxidized paper (for non-CBD-based protein assays) using hydrophobic ink as described previously.[4] Paper-based immunoassays were conducted using sequential protein addition and wicking of the flow-through. Each protein incubation step was 30 minutes unless otherwise described. Test zones were washed twice with 20 μL of 1x PBS following each incubation step.

For immobilization on non-functionalized paper, 6 μL of 30 μM of SsoZNS1.E2-CBD or ProA-CBD diluted in 1x PBS was incubated in each test zone for at least 30 seconds. For ProA-CBD assays, this step was followed by an incubation with 2 μL of rabbitAb diluted 3.33-fold in 10% glycerol in 1x PBS for 1 hour. Antibody immobilization on oxidized test zones for non-CBD-based assays occurred via overnight incubation of 2 μL of mouseAb diluted 10-fold or rabbitAb diluted 3.33-fold in 1x PBS and 10% glycerol. After overnight incubation and wash, any unreacted aldehyde sites on the functionalized paper were blocked with 10 μL of 1x TBS for one hour.

Test zones were then contacted with 10 μL of ZNS1 in PBSA (1x PBS with 1% w/v bovine serum albumin) at concentrations ranging from 0.25 nM to 512 nM. For serum studies, the biomarker

was diluted into 100% human serum from human male AB plasma (Sigma-Aldrich, H4522). For the D2NS1 cross-reactivity test, 10 μL of 1 μM of D2NS1 in PBSA was applied to each test zone. For large volume biomarker samples, 100 μL of ZNS1 in PBSA at concentrations ranging from 0.1 nM to 64 nM were flowed through the test zones by gradual application of the sample volume in 16.67 μL increments six times. After the previous applied volume passed through the well, the next aliquot of sample was added. Negative controls were conducted with the absence of biomarker (just PBSA).

For signal detection, 10 μL of 1 μM b-MBP-SsoZNS1.E1 or 5 μL of bAb diluted 16.7-fold was applied to each relevant test zone, followed by 10 μL of 1 μM SA AF647 in the dark. Samples were allowed to air-dry in the dark before imaging.

Fluorescent microscopy was used to measure level of binding, as described previously.[5] Samples were exposed for 150 ms using a Cy5 filter and imaged using Metamorph software (Molecular Devices, Sunnyvale, CA). Captured fluorescent images were processed on ImageJ (US National Institutes of Health) to determine the mean fluorescence intensity (MFI), as described previously.[5] The background (negative controls; fluorescence without presence of biomarker) plus three times the standard deviation of the negative controls (BG + 3σ) was subtracted from the samples to obtain the background adjusted MFI. Values represent an average of four replicates. Error bars indicate standard deviations.

Quadratic least squares regression and linear least squares regression were applied to each titration curve using Microsoft Excel. Linear least squares regression was conducted on the linear range, near the lower concentrations of biomarker. Using the quadratic curve fit equation, the limit of detection was calculated as the biomarker concentration at the x-intercept (where the fit curve equals zero, which is the background + 3σ). The resulting slopes from linear regression were analyzed for assessment of sensitivity differences. Error values for limit of detection and sensitivity improvement indicate standard deviations.

Protein Sequences

Red indicates biotin acceptor sequence, blue indicates MBP sequence, purple indicates rcSso7d sequence (bold and underlined amino acids comprise the binding face residues of the specific rcSso7d clone), yellow indicates CBD sequence, and green indicates protein A sequence.

b-MBP-SsoZNS1.E1

MGSSHHHHHHSSGLVPRGSHMMAGGLNDIFEAQKIEWHELKGGGGSGGGGSEFKIEEGKLVIWINGDKGY
NGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQD
KLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFT
WPLIAADGGYAFKYENGKYDIKDVGVDNSGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTIN
GPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDK
PLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTG
SGGGGSGGGGSTSATVKFTYQGEEKQVDISKIK**S**V**I**R**K**GQ**H**I**W**F**A**YDEGGGA**W**G**S**G**K**VSEKDAPKELLQM
LEKQ*

SsoZNS1.E2-CBD

MGSSHHHHHHSSGLVPRGSHMATVKFTYQGEEKQVDISKIK**R**V**Y**R**WI**GQ**D**I**G**F**I**YDEGGGA**S**G**W**G**S**VSEK
DAPKELLQMLEKQGSGGGGSGGGGSGGGGSPVSGNLKVEFYNSNPSDTTNSINPQFKVTNTGSSAIDLSK
LTLRYYYTVDGQKDQTFWCDHAAIIGSNGSYNGITSNVKGTFVKMSSSTNNADTYLEISFTGGTLEPGAH
VHIQGRFAKNDWSNYTQSNDYSFKSASQFVEWDQVTPYLNGVLVWGKEP*

ProA-CBD

MGSSHHHHHHSSGLVPRGSHMVDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAK
KLNDAQAPKVDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNGAQAPKGSG
GGGSGGGGSGGGGSPVSGNLKVEFYNSNPSDTTNSINPQFKVTNTGSSAIDLSKLTLRYYYTVDGQKDQT
FWCDHAAIIGSNGSYNGITSNVKGTFVKMSSSTNNADTYLEISFTGGTLEPGAHVHIQGRFAKNDWSNYT
QSNDYSFKSASQFVEWDQVTPYLNGVLVWGKEP*

Supplemental Data

Quadratic least squares regression

For each of the assay configurations, we analyzed the titration curves for the small (10 μL) and large (100 μL) biomarker volumes. The data points have been subtracted by the background signal (negative control; in the absence of biomarker) plus three times the standard deviation. Using quadratic least squares regression, we conducted a fitted curve analysis for each titration curve to get R^2 values close to 1. We generated the curve fit equation ($y = Ax^2 + Bx + C$, where y is the background adjusted MFI values, x is the concentration of biomarker in nM, and A, B and C are the parameters). We used the resulting fit equation to determine the x-intercept, which represents the limit of detection (nM) of each titration curve, defined as the minimum concentration at which signal can be distinguished from the background with probability.[6,7] In order to calculate the error values for the limit of detection, we fit each set of titration values of the four replicates to a quadratic fit, used the fit equations to determine the limits of detection for each replicate, and then calculated the standard deviation of the replicates. The quadratic curve fit was chosen for calculation of limits of detection because it gave better agreement with the data at the concentrations around the detection limit. The plots below represent the same data shown in the main text figures.

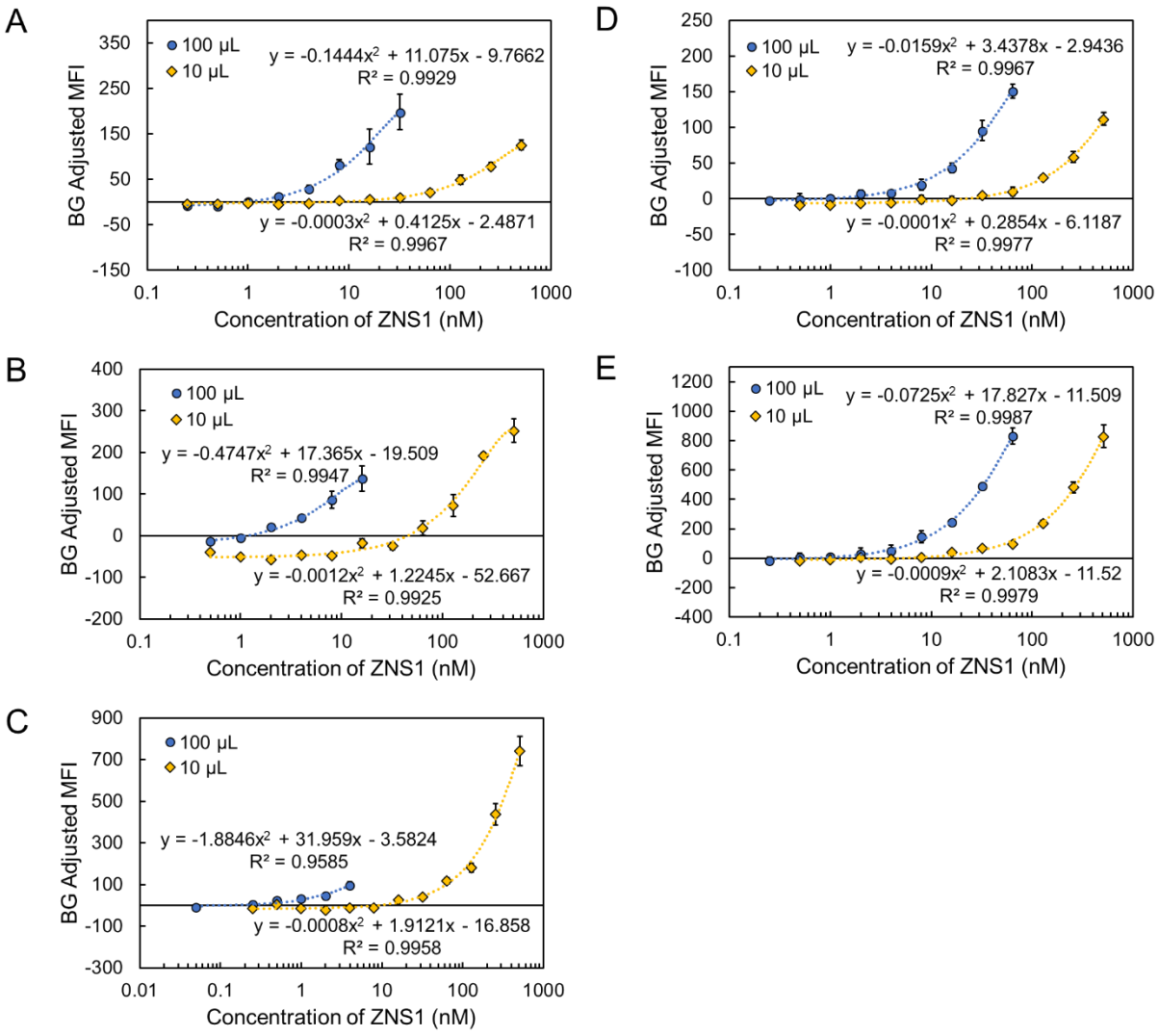

Figure S1. Titration curves for (A) rcSso7d full sandwich using rcSso7d-CBD as capture and b-MBP-rcSso7d as reporter, (B) antibody full sandwich assay using mouseAb as capture and bAb as reporter, (C) antibody full sandwich assay using rabbitAb as capture and bAb as reporter, (D) antibody-rcSso7d hybrid sandwich assay using ProA-CBD with rabbitAb as capture and b-MBP-rcSso7d as reporter, and (E) rcSso7d-antibody hybrid sandwich assay using rcSso7d-CBD as capture and bAb as reporter for ZNS1 with 10 µL (yellow) and 100 µL (blue) biomarker volumes. Curves are plotted in semi-log scale and fit to a quadratic least squares regression curve (dashed lines). The resulting curve fit equations and R^2 values are displayed on the plot. Each point represents an average of four replicates with the error bars representing the standard deviations of each point.

Linear least squares regression for assessment of sensitivity

Sensitivity of an assay can be determined by the slope of the titration curve, which explains the ability of the assay to distinguish between small changes in concentration of the biomarker.[7] For each of the assay configurations, we analyzed the titration curves for the small (10 μL) and large (100 μL) biomarker volumes. The data points have been subtracted by the background signal (negative control; in the absence of biomarker) plus three times the standard deviation. Using linear least squares regression, we conducted a fitted line through the lower concentration linear range for each titration curve to get R^2 values close to 1. We generated the curve fit equation ($y = mx + b$, where y is the background adjusted MFI values, x is the concentration of biomarker in nM, and m and b are the parameters) and used the resulting slopes (m) from the fit equation to compare the change in assay sensitivity from increase in biomarker volume from 10 to 100 μL. The values were calculated by dividing the slope of the 100 μL titration data by the slope of the 10 μL titration data. In order to calculate the error values for the fold sensitivity improvement, we fit each set of titration values of the four replicates to a linear fit, calculated the standard deviation of the slopes for the replicates, and then calculated the error propagation using the standard deviations. The plots below represent the same data shown in the above section, as well as in the main text figures, but represented on a linear scale instead of a log-based scale.

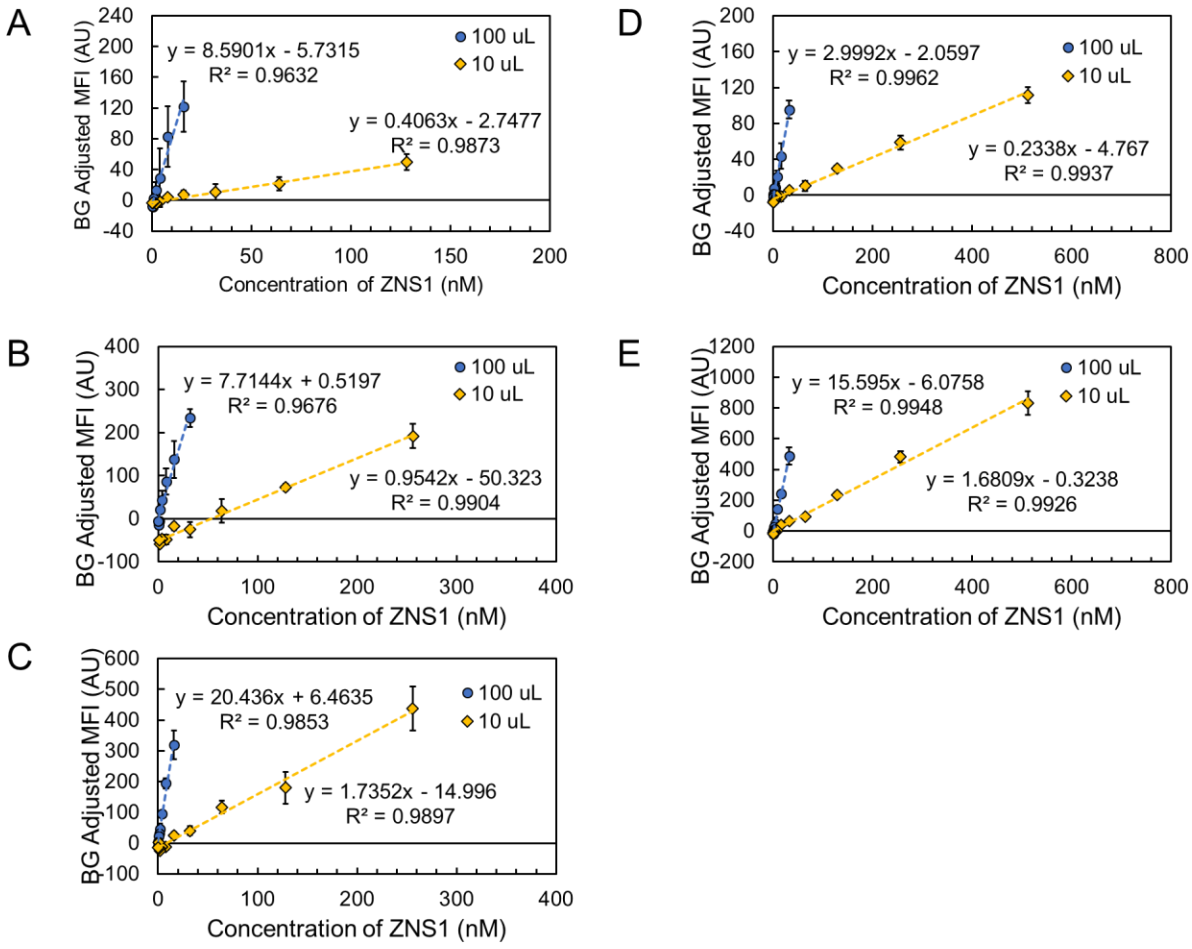

Figure S2. Titration curves for (A) rcSso7d full sandwich using rcSso7d-CBD as capture and b-MBP-rcSso7d as reporter, (B) antibody full sandwich assay using mouseAb as capture and bAb as reporter, (C) antibody full sandwich assay using rabbitAb as capture and bAb as reporter, (D) antibody-rcSso7d hybrid sandwich assay using ProA-CBD with rabbitAb as capture and b-MBP-rcSso7d as reporter, and (E) rcSso7d-antibody hybrid sandwich assay using rcSso7d-CBD as capture and bAb as reporter for ZNS1 with 10 µL (yellow) and 100 µL (blue) biomarker volumes. Curves are plotted in linear scale and fit to a linear least squares regression curve (dashed lines). The resulting curve fit equations and R^2 values are displayed on the plot. Each point represents an average of four replicates with the error bars representing the standard deviations of each point.

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