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Supplementary Materials for

Ultrasensitive point-of-care immunoassay for secreted glycoprotein detects Ebola infection earlier than PCR

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/13/588/eabd9696/DC1)

Data file S1 (Microsoft Excel format). Raw data.

Materials and Methods

sGP expression and purification

An EBOV sGP expression plasmid consisting of the mature EBOV sGP (Mayinga) through the furin cleavage site followed by a stop codon with an N-terminal human influenza hemagglutinin (HA) tag under control of the CMV promoter in pDISPLAY vector (*60*) was graciously provided by Dr. Thomas Hoenen at Rocky Mountain Laboratories. For analogous expression of sGP from other ebolavirus species, synthetic DNA gBlocks (Integrated DNA Technologies) containing SUDV, BDBV, RESTV and TAFV sGP, (genebank YP_138524, YP_003815436.1, AAV48578.1, AAB37092.1) were cloned in the pDISPLAY vector (*60*) using Gibson assembly (NEBuilder HiFi,New England Biolabs). *E. coli* containing the plasmids for each sGP were cultured overnight in Luria broth (BD Difco) with ampicillin (MilliporeSigma) at 100 µg/mL. Plasmid DNA was purified using Plasmid Plus Midi Kit (Genesee; USA) following the manufacturer's protocol. Purified DNA was Sanger sequenced (Eton Bioscience) and transfected into Expi293 cells (Thermo Fischer Scientific) with Expifectamine (Thermo Fischer Scientific), as recommended by the manufacturer. sGP proteins were purified from culture supernatants by anti-HA-tag affinity chromatography (Thermo Fischer Scientific) and stored at -80°C in 1x PBS (MilliporeSigma). Recombinant GP1 from EBOV GP1 and Marburg GP1 with a $(His)_6$ tag were purchased (Creative Diagnostics, USA).

Antigen-down ELISA

Unless stated otherwise, all reagents were obtained from MilliporeSigma**.** ELISAs were performed in Corning Costar high binding immunoassay plates. All wash steps consisted of three washes with 300 µl of phosphate buffered saline (PBS) with 0.1% (v/v) Tween 20 using a BioTek 405TS microplate washer. Plate wells were coated with 100 µl of recombinant sGP at a

concentration of 2 µg/mL in PBS by incubating the plate for 16 h at 4 °C. After washing, the wells were blocked with 200 µl of blocking solution (unless otherwise noted, 2% w/v bovine serum albumin, 5% v/v goat serum in PBS) for 2 h at room temperature. After washing, 100 µL of serial dilutions of purified antibody 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 (Thermo Fischer Scientific) diluted 1:5000 in PBS, 0.1% (v/v) Tween 20 was added. After 1 h at room temperature, the wells were washed, and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Fischer Scientific) 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 on a Spectramax M2e plate reader (Molecular Devices).

Immunizations and sera harvest

Five female WT BALB/c mice and five female WT C57BL/6 mice (8 weeks old, Charles River Laboratories) were each immunized intraperitoneally with 100 µg of EBOV sGP in a 50% slurry of alum (Invivogen). The mice were boosted with 50 µg EBOV sGP/alum intraperitoneally at 14-day intervals. Mice were periodically bled to obtain serum to assay anti-EBOV sGP IgG titers by antigen-down (Ag-down) ELISA. ELISA measurements were performed as previously described (*34*) using 1 µg/mL of sGP diluted in PBS as coating antigen. Ag-down ELISA was performed by adding serially diluted mouse serum to the plates which were incubated for 1 h on an orbital shaker. Peroxidase conjugated goat anti-mouse IgG and IgM secondary Abs (Thermo Fischer Scientific) in PBS, 0.1% Tween 20 were used for detection. After washing three times with 300 µl of 0.1% (v/v) Tween 20 in PBS using a BioTek 405TS microplate washer, o-phenylenediamine dihydrochloride (OPD), (Thermo Fischer Scientific) substrate was added. After 30 min, the reaction was stopped with 2 M sulfuric acid and the absorbance at 492 nm was

measured on a Spectramax M2e plate reader (Molecular Devices). After the anti-sGP immune titers reached a plateau, a final boost of 100 µg of EBOV sGP in a 50% slurry of alum was administered. Spleens were harvested 4 days after the terminal boost and homogenized into Trizol (Thermo Fischer Scientific).

scFv library construction

RNA was prepared from harvested spleens and reverse transcribed with Superscript III (Thermo Fischer Scientific) using random hexamer primers as previously described (*34*). Sets of gene-specific primers (*76*) (Integrated DNA Technologies) were used to join variable heavy (V_H) and variable light (V_L) fragments. Splice extension overlap was used to join V_H and V_L fragments with an intervening flexible linker, GGSSRSSSSGGGGSGGGG, and to append HindIII and SalI restriction sites for ligation at 16 °C with T4 DNA ligase (New England Biolabs) into phage display vector pAPIII(*77*). Following ligation, 30 electroporations of F' *E. coli* strain TG-1 (Lucigen) were pooled and plated on Luria-Bertani (LB) + Ampicilin (Amp) plates. The resulting colonies were scraped into LB and pooled to create library bacterial stocks from which M13 phage particles were generated by M13 K07 (New England Biolabs) rescue for selections.

Phage rescue

As described elsewhere in greater detail (*34, 78*), rescues with M13K07 (New England Biolabs) were performed to obtain M13 phage particles for selections and screening. A 100 mL LB+Amp culture was inoculated from frozen stocks and grown at 37 °C to an OD600 of 0.7. M13K07 helper phage (2×10^{11}) were added and the culture was incubated at 37 °C with no shaking for 30 min, then shaken at 200 rpm for 30 min at 37 °C. Bacteria were pelleted and the medium was replaced with 50 mL of LB+Amp supplemented with 50 µg/mL kanamycin (Kan, MilliporeSigma). The culture was shaken at 37 °C for 30 min at 200 rpm, then at 30 °C for 16 h.

The culture was centrifuged as above, and the supernatant was subjected to multiple precipitations in 0.2 volumes of ice-cold 20% polyethylene glycol 8000/2.5 M NaCl (MilliporeSigma) to purify and concentrate the phage particles. Following the last precipitation, the phage were resuspended in 1 mL PBS filtered through 0.45 µm syringe filter (VWR) and stored at 4 °C. Phage titers were determined by infections of TG-1 cells with serially diluted phage.

Immunotube phage selection

Recombinant EBOV sGP (50 µg in 1 mL PBS) was adsorbed to NUNC immunotubes (Thermo Fischer Scientific). Following adsorption, the target tube and a control tube (with no target antigen) were blocked with 4 mL of 2% (w/v) nonfat milk (Carnation) in PBS. An aliquot of 10^{11} freshly prepared M13 phage particles from the sGP scFv library was subjected to negative selection for 1 h at room temperature in the control tube coated with blocking solution, then transferred to the target tube with sGP and incubated for 1 h on a rotator. Unbound phage were removed with 20 washes of 4 mL 0.1% (v/v) in Tween 20 in PBS 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 LB+Amp 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.

Solution phase phage selection

To identify phage clones that bind to native sGP epitopes, a selection using anti-HA magnetic beads (Thermo Fischer Scientific) was performed. Beads were initially washed three times in PBS with 2% (w/v) bovine serum albumin (Calbiochem) (PBS+BSA), then incubated with 10 µg of sGP in 1 mL of PBS+BSA for 1 hour on a rotator. Unbound sGP was washed away with 3

washes of 1 mL PBS+BSA. For the first round of selection, the beads were incubated with 10^{11} phage from the anti-sGP M13 library in 1 mL of PBS+BSA for 2 hours. To remove unbound phage, five washes with 25 mL 0.1% (v/v) Tween 20 in PBS were done. Bound phage were eluted by a 10 min incubation with 1 mL 0.1 M HCl (MilliporeSigma). After neutralization, the eluted phages were used to infect TG1 host cells for plating and rescue as above. A second round of selection was done in the same fashion, using 10^{10} phage obtained from the first round.

Screening phage clones

Following selective enrichment, individual phage clones were rescued for screening in sterile 2 mL well 96-well plates (Continental Lab Products). 400 µL of LB+Amp 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 LB+Amp, supplemented with 2×10^{10} phage/mL of M13K07 helper phage. The rescue plate was shaken at 37 °C for 4 h at 200 rpm. Bacteria were pelleted at 3000*g* for 10 min, aspirated, and resuspended in 400 µL of LB + Amp + Kan, then shaken for 16 h at 30 °C at 200 rpm. The bacteria were pelleted as above, and the supernatant containing phage particles was transferred to a fresh plate for antigen-down ELISAs. Plates were coated with 100 µL of 1 µg/mL of sGP protein, then blocked with 2% (w/v) dried nonfat milk in PBS. Phage supernatants containing phage displaying scFvs were diluted with 0.1% (v/v) Tween 20 in PBS and added to the sGP coated wells. Bound phage were detected with a peroxidase-conjugated mouse IgG2a against pVIII, the major coat protein of M13 phage (GE Healthsciences). After 1 h at room temperature, the wells were washed, and 100 µl of O-phenylenediamine dihydrochloride (OPD) (Thermo Fischer Scientific) was added. After 15 min at room temperature, the reaction was stopped by adding 100 µl of 2 M sulfuric acid. Absorbance values at 492 nm were measured on a Spectramax M2e plate reader (Molecular

Devices). ScFv inserts from phage clones binding sGP for EBOV, SUDV, and BDBV with signals five-fold higher than background were amplified by PCR and Sanger sequenced (Eton Bioscience). Duplicate clones were eliminated.

Isotag purification of scFv-Fc antibodies and IgGs

Expression flasks (Nalgene) received 3.5 µM of IsoTag reagent (Isolere Bio) per mL of culture and were incubated for 10 min at 37°C. The cell cultures were then centrifuged at 14,000 rpm for 10 min at 4°C to remove cells and cellular debris. IsoTag (Isolere Bio) bound to Abs were purified from culture supernatant by two rounds of inverse transition cycling(*79*). To avoid prolonged heat exposure, IsoTag bound Abs were phase separated by the addition of 0.5 M ammonium sulfate (VWR) and centrifuged at 14,000 rpm at 37°C for 10 min. Pellets were resuspended in 1x PBS (MilliporeSigma) 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 (MilliporeSigma) pH 4, and 0.5 M ammonium sulfate (VWR) was added to trigger the phase transition. The Ab-rich supernatant was separated from the IsoTag pellet and neutralized with 0.1 mL of 1 M Tris (MilliporeSigma) pH 8.5, per mL of supernatant. Isolated Abs were buffer exchanged with 10K Amicon centrifugal ultrafiltration devices (MilliporeSigma) and concentrated to 1 mg/mL in 1x PBS (MilliporeSigma).

Binding kinetics of scFv-Fc and IgGs to sGP

Binding kinetics were determined by SPR on a Biacore T200 instrument (GE Healthcare). scFv-Fc and IgG solutions at 1 µg/mL concentration in HBS-EP running buffer (GE Healthcare) were injected on to a protein A sensor chip (GE Healthcare) at a 5 µl min^{-1} rate until the final resonance units (RU) of approximately 350 was reached. For scFv-Fc's, varying concentration of EBOV sGP were prepared as a 2-fold dilution in HBS-EP running buffer (GE Healthcare) and

were injected at a 30 µl min^{-1} flow rate for 200 s, and dissociation was monitored for 600 s. For A1F3-1 and C2BA5-2 IgGs, varying concentrations of EBOV, SUDV, BDBV, RESTV and TAFV sGPs were injected at a 30 µl min^{-1} flow rate for 200 s, and dissociation was monitored for 600 s. Following each injection round of scFv-Fc or IgG and sGP, the chip surface was regenerated with two sequential 30 s injections of 10 mM of glycine-HCl pH 2 buffer (GE Healthcare) at a 30 µl min^{-1} flow rate. Experimental data for Ab/Ag interaction were fit with a 1:1 binding model using global fits and local R_{max} with BIA evaluation software (GE Healthcare). The only exception to this methodology was for the SPR sensorgram of A1F3-1 and RESTV sGP, which was fit with a two-state reaction model with global fits as the sensorgram indicated a conformational change after the binding event incompatible with the 1:1 binding model.

POEGMA growth on SiO_2 and glass surfaces

Unless stated otherwise, all reagents were obtained from MilliporeSigma. As described elsewhere in greater detail (*29*) and summarized here, Nexterion B Glass slides (SCHOTT) and silicon wafers with a thermally grown oxide layer (University Wafer) were immersed in a 10% (v/v) solution of (3-aminopropyl)triethoxysilane (APTES) (Gelest) in ethanol (KOPTEC) for 4 h. Following an ethanol (KOPTEC) and deionized (DI) water rinse, substrates were centrifuged at 150 rcf on an Allegra X-15R Centrifuge (Beckman Coulter) for 6 min, and cured overnight at 120 °C. After cooling, substrates were immersed in a solution of 1% (v/v) α-bromoisobutyryl bromide (BiB) and 1% (v/v) triethylamine in dichloromethane (VWR). Following incubation for 30 min, substrates were rinsed with fresh dichloromethane (VWR), ethanol (KOPTEC), DI water and centrifuged at 150 rcf for 6 min in a Allegra X-15R centrifuge (Beckman Coulter), leading to surfaces functionalized with BiB — the ATRP initiator (termed APTES-BiB). Next, a solution

composed of 50 g of POEGMA (inhibitor-free), 28 mg of copper(II) bromide, 100 μL of 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA), and 350 mL of deionized (DI) water was degassed by sparging with helium for 3 h. Under an argon environment, 600 mg of sodium ascorbate was added to the degassed solution. Following the expected color change from blue to violet, the BiB-functionalized glass slides were immersed in the polymerization solution for 5 h. Next, they were rinsed with DI water, spun dry for 6 min at 150 rcf in a Allegra X-15R Centrifuge (Beckman Coulter) and stored under ambient conditions for subsequent use. Film thickness was measured with an M-88 spectroscopic ellipsometer (J.A. Woollam Co) at wavelengths of 400 to 800 nm and angles of 65, 70 and 75° and was calculated by fitting the change in light polarization to a Cauchy model with optical constants provided with the VASE software (J.A. Woollam Co)(*24*, *29*).

D4 assay accelerated fluorescence output stability

Chips were incubated with EBOV sGP spiked MoS for 15-, 60- and 90-min, and were then scanned immediately after the rinse step. They were then stored protected from direct light exposure under ambient conditions (~25°C, 40% humidity) for ten days prior to being rescanned. Chips tested with EBOV sGP spiked FBS (Avantor) after 90 min incubation were also scanned at Days 0, 10 and 60 while stored under ambient conditions protected from light. One set of negative controls —chips incubated with FBS— were stored within aluminum pouches (EASE Medtrend) with 5 g silica bags (EASE Medtrend) and exposed to an environment of 37 °C and 100% humidity. These chips were scanned at Days 0, 15 and 30. Another set of chips run with EBOV sGP-spiked FBS were also stored within aluminum pouches (EASE Medtrend) with 5 g silica bags (EASE Medtrend) immediately after being scanned. Following exposure to 37°C for 15 days, the chips were rescanned and then exposed to 37°C and 100% humidity for 12 days

(outside the aluminum pouch), when they were scanned again. Finally, a set of chips run with EBOV sGP in HS (Innovative Research) were stored at 37°C and 50% relative humidity and scanned at Days 0 and 10. Arrays were imaged and quantified with the same Axon Genepix 4400 (Molecular Devices) scanner with a photomultiplier gain of 750 and excitation power of 100. As previously described, the LoD and DR were determined for each set of samples at the specified time points.

Lateral flow assay fabrication and performance evaluation

Unless stated otherwise, all reagents were obtained from MilliporeSigma. To fabricate an LFA for sGP, first, we synthesized gold nanoparticles (GnPs) with a citrate reduction technique described elsewhere in greater detail (*80*). In summary, 20 mL of a 1% (w/v) gold (III) chloride trihydrate stock solution was added to 1000 mL of DI water. This solution was brought to a boil under reflux and 20 mL of a 4% (w/v) trisodium citrate dihydrate stock solution was added under vigorous agitation. After 20 min under reflux, the heat was turned off and the bright orange-red solution was left to cool overnight and was stored at 4°C. The newly synthesized GnPs were directly conjugated to dAb (C2bA5-2) by chemisorption of the dAb via thiol groups in solvent exposed Cys residues of the Ab to the GNP as follows. First, the dAb was buffer exchanged into a 20 mM HEPES HCl buffer, pH 7.4 and concentrated to 1 mg/mL by centrifugal ultrafiltration with a 10kDa Amicon MWCO spin filter. Next, 10 mL of a 200 mM HEPES HCl buffer, pH 7.4 was added to 90 mL of GnP colloidal suspension to adjust its pH to 7.4. Then, a dAb dilution series from 0.5 mg $mL^{-1}$to 0.002 mg/mL was made from the 1 mg/mL Ab stock solution with 20 mM HEPES. To identify the optimal GnP conjugation conditions, 100 µl of the pH adjusted GnP suspension was added to 100 µl of each aliquot of the dilution series, following which 100 µl of a 2 M NaCl solution was added. The ratio of antibody to GnP suspension that maintains its

original color (red), indicates the optimal Ab amount to be conjugated. Using this ratio, 1 mL of the 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 colloidal suspension under vigorous stirring. Following conjugation, the GnP-dAb suspension was left under mild stirring for 10 min. Next, 10 mL of bovine serum albumin (20%) and sodium azide (1% w/v) were added under mild agitation, followed by 10 mL of 10x PBS. This final GnP-dAb conjugate suspension was stored overnight at 4 °C.

Following conjugation, GnP-dAb were lyophilized on fiber glass membranes to generate the conjugate pads. To do so, sucrose was added to a final concentration of 2% (w/v) and 200 µl of the GnP-dAb conjugate was evenly distributed per 1 cm^2 of membrane on fiberglass sheets (EASE-Medtrend) that had previously been washed with 0.01% (v/v) Tween 20 in DI water and air-dried overnight. The conjugate suspension infused membranes were then flash-frozen in liquid nitrogen and lyophilized for 72 h in a Labconco Freeze Dryer (Labconco). Following lyophilization, samples were removed and immediately stored under vacuum to avoid exposure to moisture.

FF80HP nitrocellulose membranes (GE Healthcare) were printed with reagents using an AD1520 non-contact dispenser (Biodot). Test lines of the cAb (A1F3-1), at 1 mg/mL concentration with 0.05% (w/v) trehalose, were printed at a dispensing rate of 1 µl of solution per linear cm. Control lines of goat-anti mouse antibodies (American Qualex Antibodies) at a 0.5 mg/mL concentration with 0.05% (w/v) trehalose, were printed at a dispensing rate of 1 µl of solution per linear cm. Following printing, membranes were left to dry overnight under mild vacuum in a vacuum dessicator.

In a controlled humidity environment (<30% relative humidity), the nitrocellulose with printed reagents, MF1 RBC filtering membranes (GE Heatlhcare), wicking pads (EASE-Medtrend), 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 then placed into plastic cassettes (EASE-Medtrend) with sample port and test and control line references. Next, plastic cassettes were placed into aluminum pouches (EASE-Medtrend) with 0.5 g silica desiccant bags (EASE-Medtrend) and heat sealed for long term storage.

The performance of the LFA was assessed by adding 25 µL aliquots of FBS (Avantor), HS (Innovative Research) and WHB (Innovative Research) with different concentrations of EBOV sGP to the LFA's through the sample port, immediately after, 3 drops of a running buffer with 0.1% (v/v) Tween-20 in PBS were added. Following 15, 60 and 90 min incubation, the LFA's were read by two different users to ensure accurate results. Assay performance was determined by running 3 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. It was determined that samples with 31 ng/mL were weakly positive with a 15 min assay time and positive with a 60 min assay time. 8 ng/mL samples were negative with an assay time of 15 min, but weakly positive with an incubation time of 60 and 90 min. 4 ng/mL samples were always negative independent of the incubation time. To confirm the 6 ng/mL LOD, samples with this concentration were added to 5 test devices. All became weakly positive within 60 min of incubation.

Anti-GP antibody ELISA

Unless stated otherwise, all reagents were obtained from MilliporeSigma. Serum from Ebola Zaire infected cynomolgus macaques was inactivated by gamma irradiation (~5 mrad) prior to analysis for immunoglobulin G (IgG) antibodies against Ebola Zaire. ELISA with recombinant glycoprotein minus the transmembrane domain (GPdTM, Integrated BioTherapeutics, Inc.) was used to detect cross-reactive IgG. Species specific GPdTM were diluted to an optimal working concentration of 0.08 µg/mL and used to coat the 96 well ELISA plates (Nunc). The serum samples were assayed at 2-fold dilutions starting at a 1:100 dilution in ELISA diluent composed of 1% heat inactivated fetal bovine serum (HI-FBS), PBS, and 0.2% Tween-20. Samples were incubated for 1 h at room temperature, and the plates were then washed three times with 300 µl of 0.1% (v/v) Tween 20 in PBS using a BioTek 405TS microplate washer. Wells were then incubated at room temperature for 1 h with anti-monkey IgG conjugated to horseradish peroxidase (Fitzgerald Industries International) at a 1:2500 dilution. The wells were then washed three times with 300 µl of 0.1% (v/v) Tween 20 in PBS and then incubated with 2,2'-azine-di(3ethylbenzthiazoline-6-sulfonate) peroxidase substrate (KPL) and read for dilution endpoints at 405 nm on a microplate reader (Molecular Devices Emax system).

Western blots

Nonhuman primate serum was inactivated with an equal volume of 4X Laemmli sample buffer (250 mM Tris-HCl, pH 6.8; 40% glycerol; 1.43 M β-mercaptoethanol; 0.02% bromophenol blue; 8% SDS) by heating to 95°C for 10 min. Similarly, recombinant viral EBOV-GP standard (R&D Systems, Minneapolis, MN) and sGP standard were diluted using radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 1% NP-40 substitute; 1% sodium

deoxycholate; 0.1% SDS) and treated with an equal volume of 4X Laemmli sample buffer and heated to 95 ^{o}C for 10 min.

For each sample, 35 µl was run alongside Precision Plus Protein WesternC ladder (Bio-Rad Laboratories Inc) on a denaturing 8-16% polyacrylamide Novex WedgeWell precast gel (Thermo Fischer Scientific) at 100 V and then transferred to a nitrocellulose membrane using the Power Blotter System at 25 V and 1.3 A for 7 min (Thermo Fischer Scientific). Membranes were blocked in Tris-buffered saline with 0.1% (v/v) Tween 20 5% (w/v) BSA (Fisher Scientific) for 1 h. Subsequently, membranes were incubated with the C2BA5 antibody against GP/sGP at a final concentration of 1 µg/mL in TBST buffer with 5% BSA overnight at 4 °C. Following four washes in TBST, membranes were incubated with Pierce goat anti-mouse IgG heavy and light chain conjugated to horseradish peroxidase (HRP; dilution, 1:5,000; Thermo Fischer Scientific) and Precision Protein StrepTactin-HRP Conjugate (dilution, 1:10,000; Bio-Rad) for 2 hours at room temperature. Membranes were washed four more times in TBST and were then incubated with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fischer Scientific) for 2 min and imaged on an iBright FL1500 Western Blot imaging system (Thermo Fischer Scientific) (*81*).

Supplementary Figures

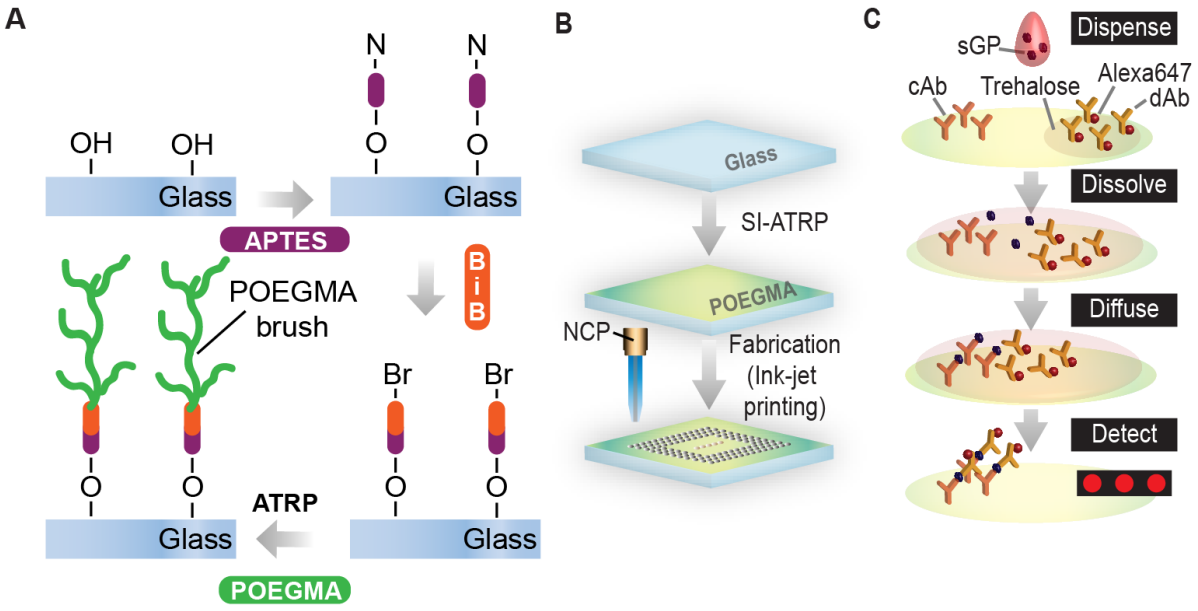


Fig. S1. Schematic depiction of EBOV D4 assay. (A) POEGMA brush growth on thermally deposited SiO_2 on Si wafers and glass slides. Surfaces are functionalized with APTES, followed by installation of ATRP initiator BiB. POEGMA brushes are then grafted from the BiB-functionalized surface by SI-ATRP in an aqueous environment. **(B)** D4 assay fabrication workflow, where POEGMA brushes are grafted onto the substrate surface by SI-ATRP, followed by non-contact printing (NCP) of cAbs in the central region of the chip and FL-dAb in a concentric pattern around the cAbs. Following mild desiccation, the D4 assay chips are ready to be used. **(C)** Cartoon of EBOV D4 assay, where sample containing sGP is dispensed onto the chip's surface. The aqueous solvent dissolves an underlying trehalose pad and the trehalose that is co-printed with the fluorescently labeled dAb, which leads to liberation of the fluorescently labeled dAb from the surface into the solution phase. The fluorescently labeled dAb subsequently diffuses on the chip's surface and binds the sGP bound to the capture Abs. Following a rinse step, the fluorescence signal from the capture spots is imaged by a tabletop

fluorescence scanner or a handheld fluorescence reader—the D4Scope. EtOH, ethanol; APTES, 3-aminopropyl-triethoxysilane; BiB, α-bromoisobutyryl bromide; DCM, diclhoromethane; POEGMA, poly(oligo(ethylene glycol) methyl ether methacrylate); SI-ATRP, surface-initiated atom transfer radical polymerization; NCP, non-contact printer; cAb, capture antibody; dAb, detection antibody; sGP, secreted glycoprotein.

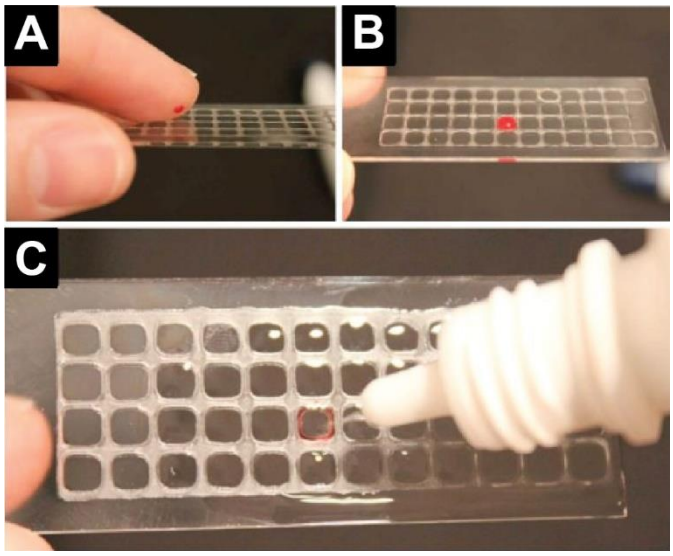


Fig. S2. D4 assay procedure. Adapted from Joh *et al. (24).* **(A)** Photograph of glass slide with D4 assay printed on its surface receives sample of interest. **(B-C)** Photographs of samples incubated (B) and rinsed with wash buffer aided by a dropper bottle **(C)**. To accommodate safety protocols in BSL-4 lab, D4 assay chips were inserted in containers with wash buffer instead of being rinsed with a dropper bottle.

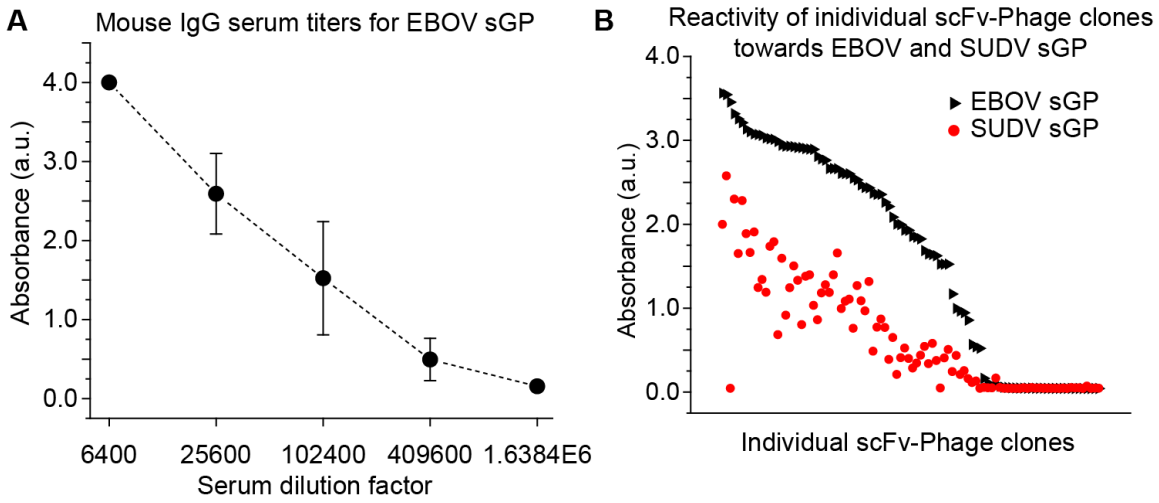
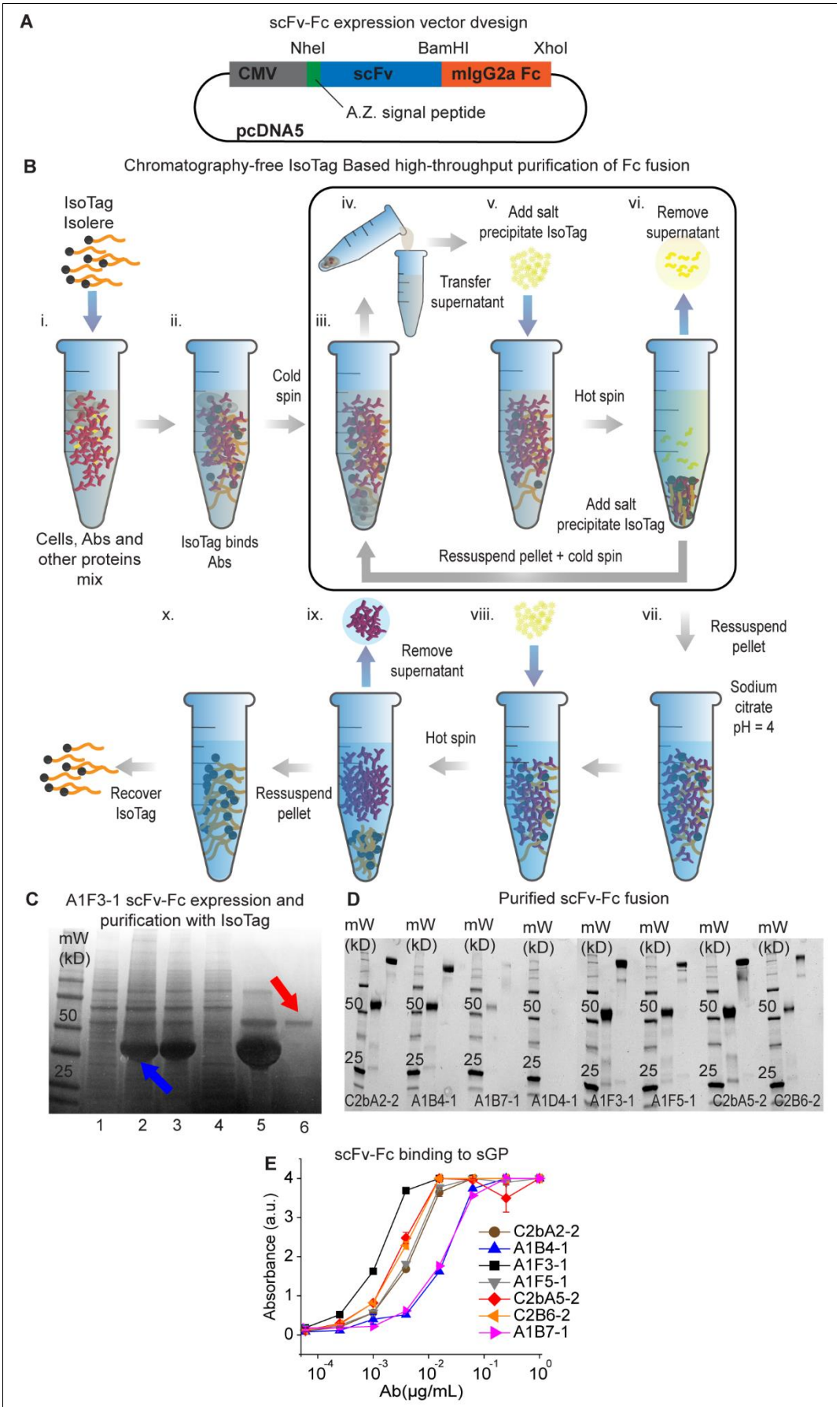


Fig. S3. Mouse IgG titers for sGP and reactivity of individual scFv-phage clones for EBOV and SUDV sGP. (A) Mouse IgG serum titers for EBOV sGP. Antigen-down ELISA for mice immunized with sGP preparations following terminal bleed. Each data point is the mean ± SD of measurements performed on n = 10 mice. **(B)** Binding profiles of scFv-phage clones to EBOV sGP and SUDV sGP evaluated using antigen-down ELISA. Each data point pair (black and red) represents N = 1 measurement of n = 1 scFv-phage clone with distinct sequences.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scFv-Fc expression vector dvesign
NheI
BamHI
XhoI
CMV
scFv
mIgG2a Fc
A.Z. signal peptide
pcDNA5
B
Chromatography-free IsoTag Based high-throughput purification of Fc fusion
IsoTag Isolere
i.
ii.
iii.
iv.
v.
Add salt precipitate IsoTag
vi.
Remove supernatant
Transfer supernatant
Cold spin
Hot spin
Add salt precipitate IsoTag
Cells, Abs and other proteins mix
IsoTag binds Abs
Ressuspend pellet + cold spin
vii.
Ressuspend pellet
Sodium citrate pH = 4
viii.
Hot spin
ix.
Remove supernatant
x.
Ressuspend pellet
Recover IsoTag
C
A1F3-1 scFv-Fc expression and purification with IsoTag
mW (kD)
50
25
1
2
3
4
5
6
D
Purified scFv-Fc fusion
mW (kD)
50
25
C2bA2-2
A1B4-1
A1B7-1
A1D4-1
A1F3-1
A1F5-1
C2bA5-2
C2B6-2
E
scFv-Fc binding to sGP
Absorbance (a.u.)
Ab(μg/mL)
C2bA2-2
A1B4-1
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A1F5-1
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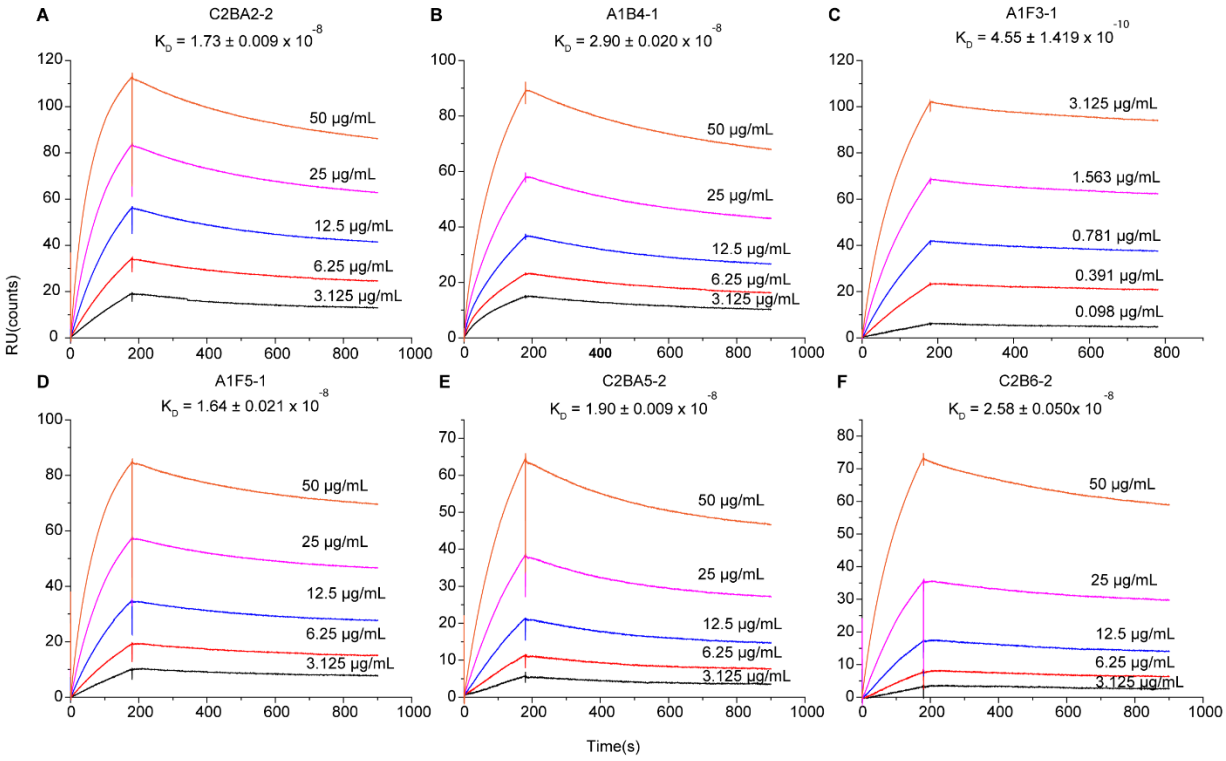
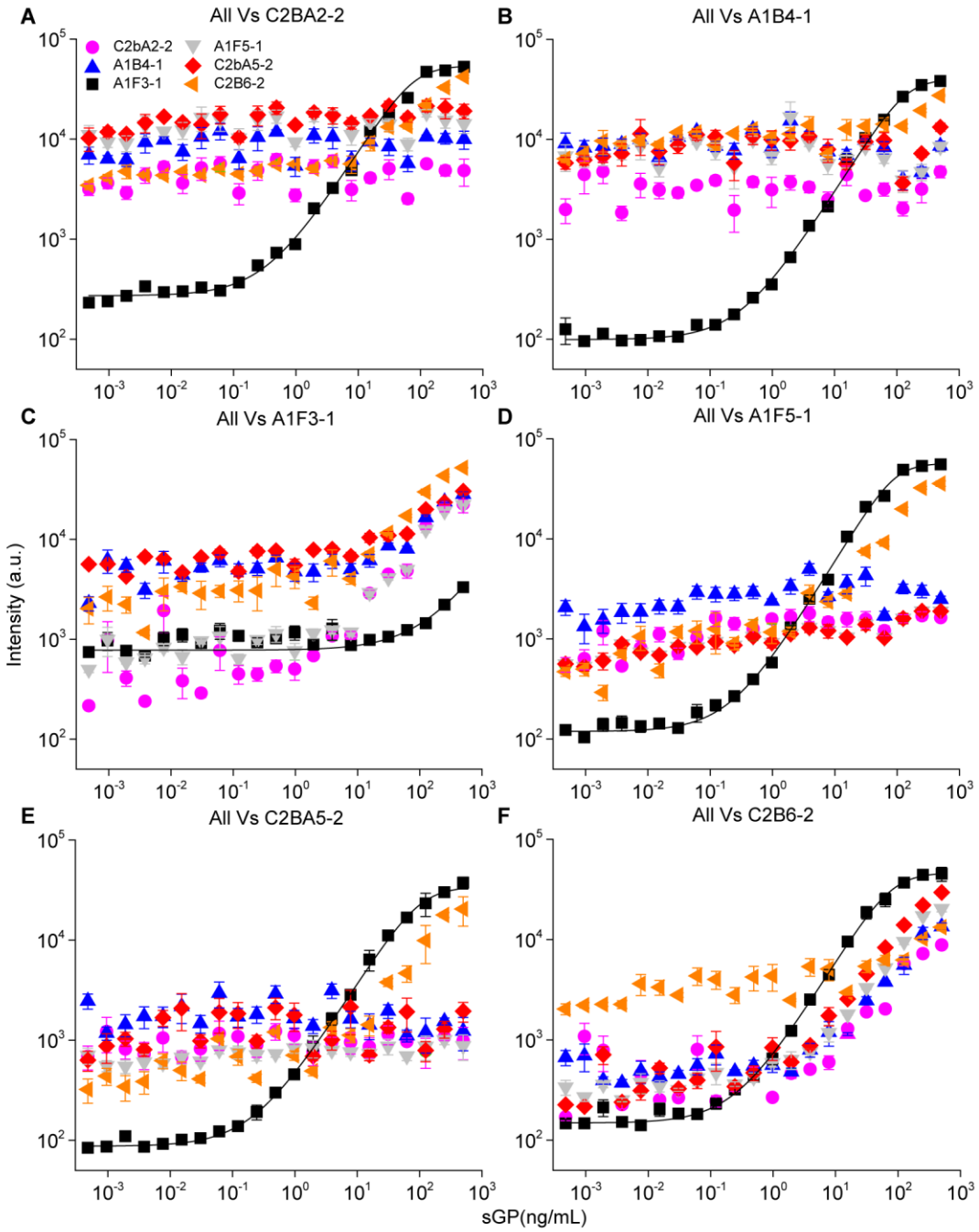
C2B6-2
A1B7-1

Fig. S4. scFv-Fc Ab expression, purification, and activity. (A) pcDNA5 scFv-Fc expression vector with cytomegalovirus promoter, mouse IgG2a Fc fusion, and azurocidin (AZ) signal peptide. **(B)** Schematic of IsoTag based Fc-fusion purification. (i) IsoTag solution is added to the expression media. (ii) IsoTag binds mIgG2a Fc. (iii) A cold-spin pellets cellular debris. (iv) Supernatant containing soluble proteins is recovered. (v) 0.4 M ammonium sulfate is added triggering phase-transition of the IsoTag bound to the Fc-fusion protein, which becomes insoluble. (vi) Following centrifugation (hot spin) the supernatant is discarded, and the pellet is resuspended in PBS. This process from (iii) until (vi) is repeated until the desired purity level is achieved. (vii) The pellet is then resuspended in sodium citrate buffer at pH 4. (viii) A hot-spin is performed after ammonium sulfate is added. (ix) The supernatant is recovered and contains the purified Fc fusion protein. (x) IsoTag is recovered and can be later reused. **(C)** SDS-PAGE showing isolation of A1F3-1 scFv-Fc using IsoTag. 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) hot-spin supernatant; (5) resuspended pellet following a second cold-spin; (6) purified scFv-Fc construct run using reducing conditions. IsoTag is indicated by a blue arrow; purified scFv-Fc by a red arrow. **(D)** SDS-PAGE of purified scFv-Fc fusions. Lanes 1 and 2 for each construct show purified scFv-Fc under (1) reducing and (2) non-reducing conditions. **(E)** Binding profiles of seven scFv-Fc antibodies to EBOV sGP evaluated using antigen-down ELISA. Each data point represents the mean ± SEM of two independent runs. CMV, cytomegalovirus promoter; MW, molecular weight; kD, kilodalton.



G	Binding Constants		
Construct	k_{on}	k_{off}	K_D
C2BA2-2	$2.23 \pm 0.006 \times 10^{4}$	$3.84 \pm 0.026 \times 10^{-4}$	$1.73 \pm 0.009 \times 10^{-8}$
A1B4-1	$1.37 \pm 0.013 \times 10^{4}$	$3.98 \pm 0.010 \times 10^{-4}$	$2.90 \pm 0.020 \times 10^{-8}$
A1F3-1	$1.86 \pm 0.018 \times 10^{5}$	$0.90 \pm 0.327 \times 10^{-4}$	$4.55 \pm 1.419 \times 10^{-10}$
A1F5-1	$1.73 \pm 0.006 \times 10^{4}$	$2.84 \pm 0.032 \times 10^{-4}$	$1.64 \pm 0.021 \times 10^{-8}$
C2BA5-2	$1.74 \pm 0.006 \times 10^{4}$	$3.30 \pm 0.029 \times 10^{-4}$	$1.90 \pm 0.009 \times 10^{-8}$
C2B6-2	$1.10 \pm 0.012 \times 10^{4}$	$2.83 \pm 0.044 \times 10^{-4}$	$2.58 \pm 0.050 \times 10^{-8}$

Fig. S5. Binding constants of scFv-Fc Abs for sGP. SPR sensorgram for binding of C2BA2-2 **(A)**, A1B4-1 **(B),** A1F3-1 **(C),** A1F5-1 **(D),** C2BA5-2 **(E),** and C2B6-2 **(F)** to EBOV sGP. scFv-Fc antibodies were bound to Protein A chips and solutions of EBOV sGP at different concentrations were injected over the chip. **(G)** Binding rate constants were determined using global fits and local R_{max} fit of three independent sensorgrams based on the 1:1 reaction model. K_D, equilibrium dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant. Each data point represents the mean ± SEM of $N = 3$ independent measurements.



G **scFv-Fc Pairing by D4 Assay**

Detection	C2BA2-2		A1B4-1		A1F3-1		A1F5-1		C2BA5-2		C2B6-2	
Constructs / Capture	LoD (ng/mL)	DR (log10)	LoD (ng/mL)	DR (log10)	LoD (ng/mL)	DR (log10)	LoD (ng/mL)	DR (log10)	LoD (ng/mL)	DR (log10)	LoD (ng/mL)	DR (log10)
C2BA2-2	-	-	-	-	8.34	1.77	-	-	-	-	17.40	1.45
A1B4-1	-	-	-	-	28.68	1.24	-	-	-	-	30.31	1.21
A1F3-1	0.31	3.2	0.24	3.3	30.1	1.22	0.40	3.1	0.13	3.6	0.27	3.27
A1F5-1	-	-	-	-	3.21	2.2	-	-	-	-	1.51	2.5
C2BA5-2	-	-	-	-	6.58	1.88	-	-	-	-	2.85	1.88
C2B6-2	1.35	2.6	72.64	0.8	72.64	0.8	7.91	1.80	-	-	417.46	0.8

Fig. S6. Ab pairing dose-response curves. (A-F) Dose-response curves of C2BA2-2, A1B4-1, A1F3-1, A1F5-1, C2BA5-2 and C2B6-2 scFv-Fc antibodies as cAb versus fluorescently labeled C2BA2-2**(A)**, A1B4-1**(B)**, A1F3-1**(C)**, A1F5-1**(D)**, C2BA5-2**(E)**, and C2B6-2**(F)** as dAb. Each data point represents the mean ± SEM of $N = 4$ independent D4 assays. Dose-response curves in black are for A1F3-1 as cAb. Dose-response curves were fitted using a 5-parameter logistic fit. **(G)** LoD and DR for each pair. "-" indicates not evaluated due to lack of dose-response behavior.

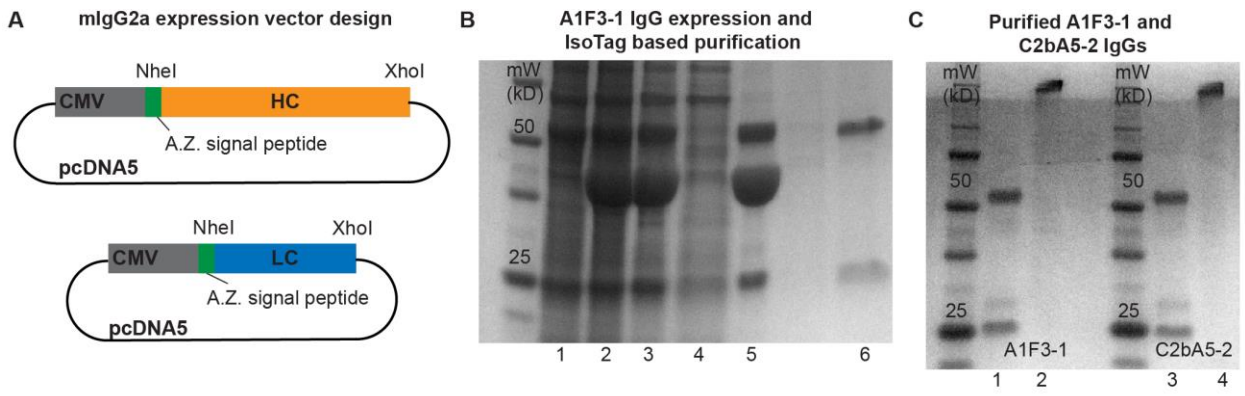
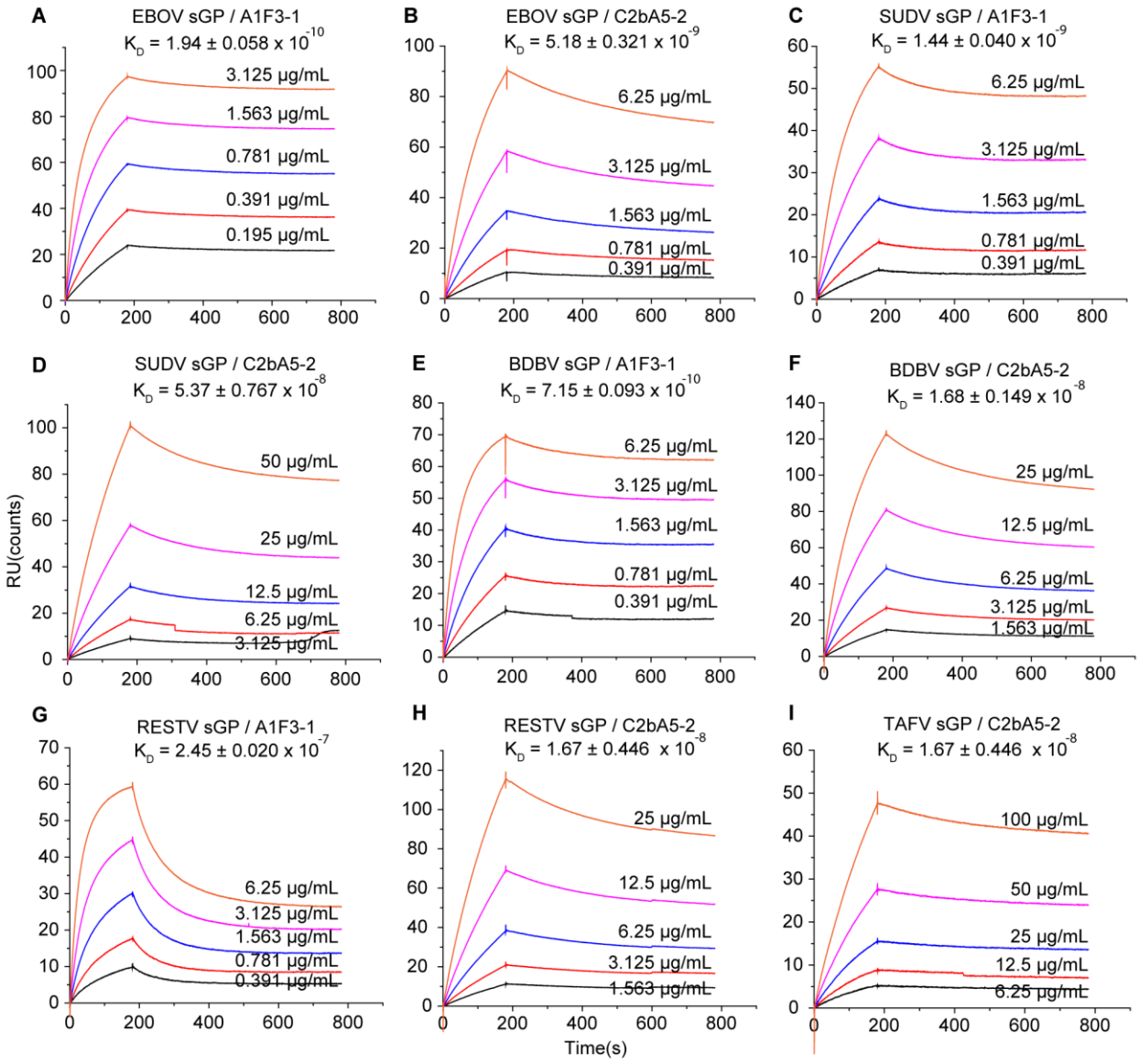


Fig. S7. Expression and purification of A1F3-1 and C2bA5-2 monoclonal IgG2a Abs. (A) pcDNA5 expression vector with cytomegalovirus promoter and azurocidin (AZ) signal peptide. **(B)** SDS-PAGE showing A1F3-1 purification using IsoTag. Lanes 1-6: (1) cell culture extract prior to addition of IsoTag; (2) cell culture extract after addition of IsoTag; (3) cold-spin supernatant, (4) RT-spin supernatant; (5) resuspended pellet following an extra cold spin; (6) purified mAb run under reducing conditions. **(C)** SDS-PAGE of purified A1F3-1 and C2BA5-2. Lanes 1 and 3: purified antibodies using reducing conditions; lanes 2 and 4: non-reducing conditions. HC, heavy chain; LC, light chain; CMV, cytomegalovirus; AZ, azurocidin; MW, molecular weight; kD, kilodalton.

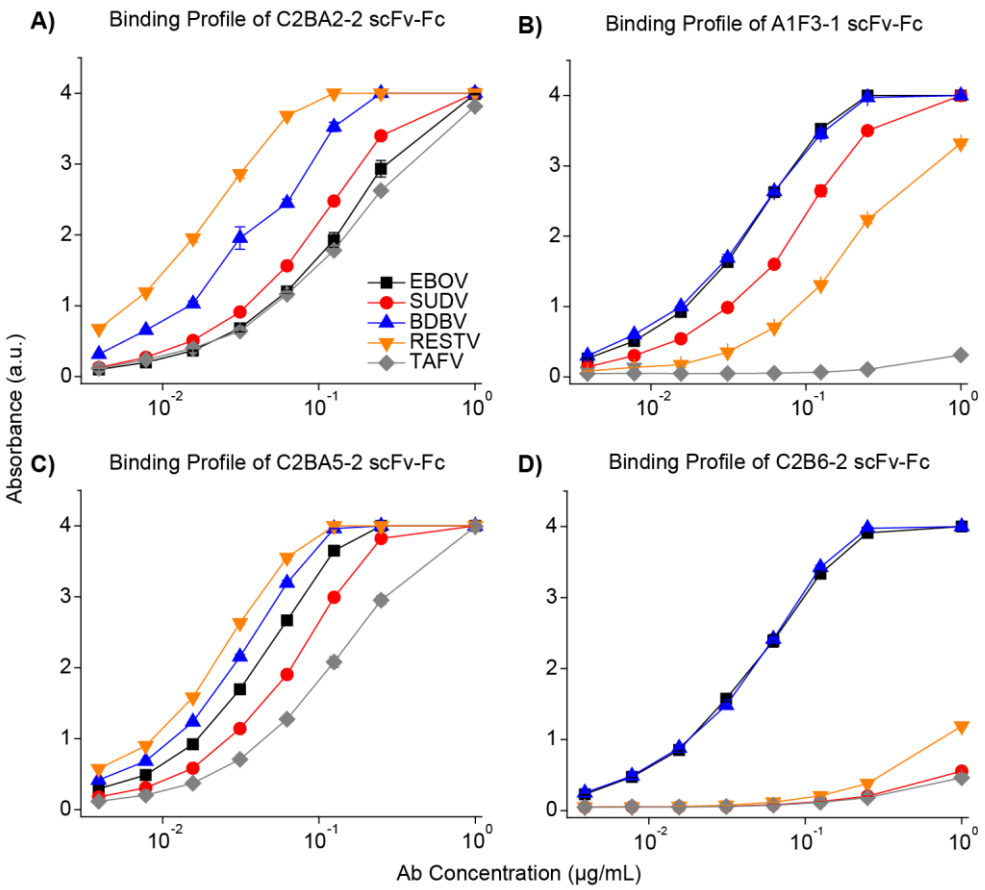


J **Mouse IgG2a Constructs**

Binding Constants

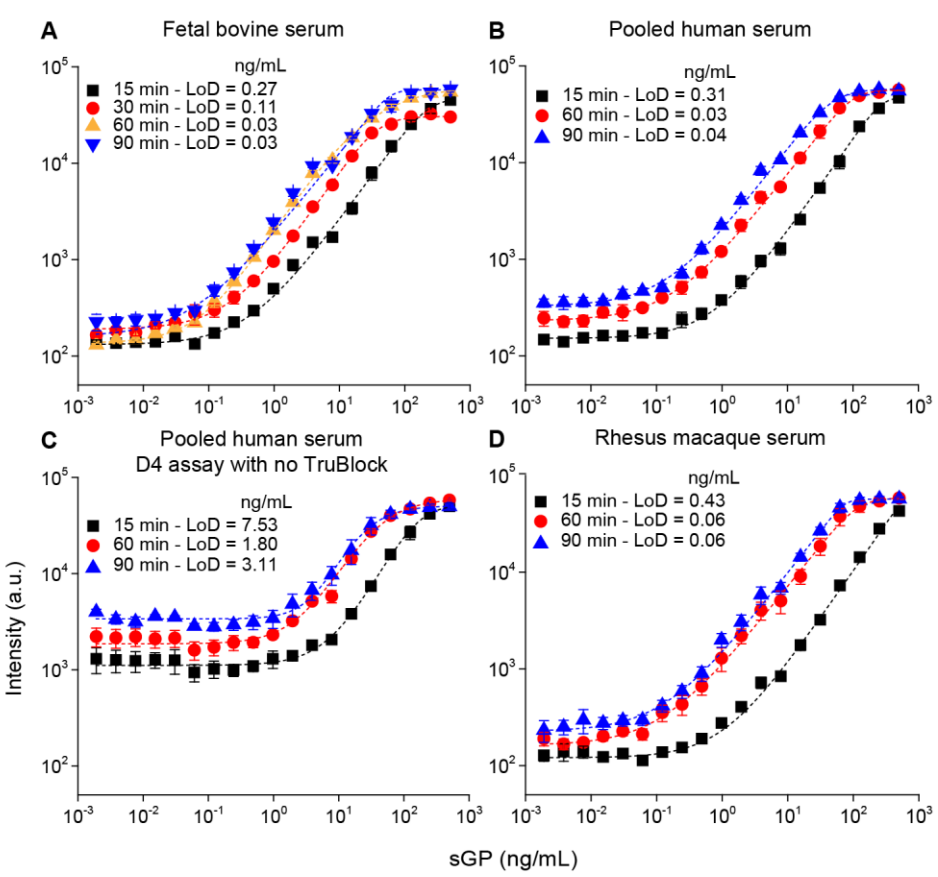
sGP	Construct	k_{on}	k_{off}	K_D
EBOV	A1F3-1	$4.91 \pm 0.012 \times 10^{5}$	$0.96 \pm 0.030 \times 10^{-4}$	$1.94 \pm 0.058 \times 10^{-10}$
	C2bA5-2	$8.66 \pm 0.647 \times 10^{4}$	$4.44 \pm 0.052 \times 10^{-4}$	$5.18 \pm 0.321 \times 10^{-9}$
SUDV	A1F3-1	$1.42 \pm 0.318 \times 10^{5}$	$2.04 \pm 0.100 \times 10^{-4}$	$1.44 \pm 0.040 \times 10^{-9}$
	C2bA5-2	$0.99 \pm 0.246 \times 10^{4}$	$4.98 \pm 0.370 \times 10^{-4}$	$5.37 \pm 0.767 \times 10^{-8}$
BDBV	A1F3-1	$2.37 \pm 0.053 \times 10^{5}$	$1.70 \pm 0.024 \times 10^{-4}$	$7.15 \pm 0.093 \times 10^{-10}$
	C2bA5-2	$3.21 \pm 0.390 \times 10^{4}$	$5.26 \pm 0.241 \times 10^{-4}$	$1.68 \pm 0.149 \times 10^{-8}$
RESTV	A1F3-1	$7.09 \pm 0.049 \times 10^{4}$	$1.70 \pm 0.023 \times 10^{-2}$	$2.45 \pm 0.020 \times 10^{-7}$
	C2bA5-2	$5.07 \pm 1.568 \times 10^{4}$	$7.07 \pm 0.900 \times 10^{-4}$	$1.67 \pm 0.446 \times 10^{-8}$
TAFV	A1F3-1	-	-	-
	C2bA5-2	$3.63 \pm 0.308 \times 10^{3}$	$0.26 \pm 0.023 \times 10^{-4}$	$7.29 \pm 0.613 \times 10^{-8}$

Fig. S8. Binding constants of A1F3-1 (cAb) and C2bA5-2 (dAb) with multiple sGPs. Sensorgram of A1F3-1 and C2bA5-2 binding to sGP from EBOV **(A-B)**, SUDV **(C-D)**, BDBV **(E-F)**, RESTV **(G-H)**, and TAFV **(I).** A1F3-1 does not bind to sGP from TAFV. IgGs were bound to Protein A chips and samples with different sGP concentrations were injected. **(J)** Binding constants were determined using global fits and local R_{max} of three independent sensorgrams based on the Langmuir (1:1) reaction model. The binding constants of RESTV sGP to A1F3-1 were modeled using global fits based on the two-state reaction model. K_D, equilibrium dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant. Each data point represents the mean ± SEM of $N = 3$ independent measurements.



E)	sGP Binding Profile			
Ebolavirus	C2BA2-2	A1F3-1	C2BA5-2	C2B6-2
Zaire	+	+	+	+
Sudan	+	+	+	-
Bundibugyo	+	+	+	+
Reston	+	+	+	-
Tai Forest	+	-	+	-

Fig. S9. Binding profiles of other scFv-Fc Abs with sGP. Binding profile of C2BA2-2 **(A)**, A1F3-1 **(B),** C2BA5-2 **(C)** and C2B6-2 **(D)** to EBOV, SUDV, BDBV, RESTV, and TAFV sGP evaluated using antigen-down ELISA. Each data point represents the mean ± SEM of N = 2 technical replicates. **(E)** Summary of binding profiles of antibodies to multiple sGPs. "+" indicates effective binding, "-" indicates lack of effective binding.



E **D4 assay performance**

i.

Incubation (minutes)	Calf serum LoB (ng/mL)	Calf serum LoD (ng/mL)	Calf serum DR (log10)	Human serum LoB (ng/mL)	Human serum LoD (ng/mL)	Human serum DR (log10)	Macaque serum LoB (ng/mL)	Macaque serum LoD (ng/mL)	Macaque serum DR (log10)	Human serum (no TruBlock D4) LoB (ng/mL)	Human serum (no TruBlock D4) LoD (ng/mL)	Human serum (no TruBlock D4) DR (log10)
15	0.10	0.27	3.27	0.10	0.31	3.21	0.16	0.43	3.07	4.54	7.53	1.82
60	0.004	0.03	4.28	<0.001	0.03	4.21	0.004	0.06	3.96	1.14	1.80	2.44
90	0.02	0.03	4.19	<0.001	0.04	4.12	<0.001	0.06	3.94	1.45	3.11	2.21

ii. 8 Log dose-response

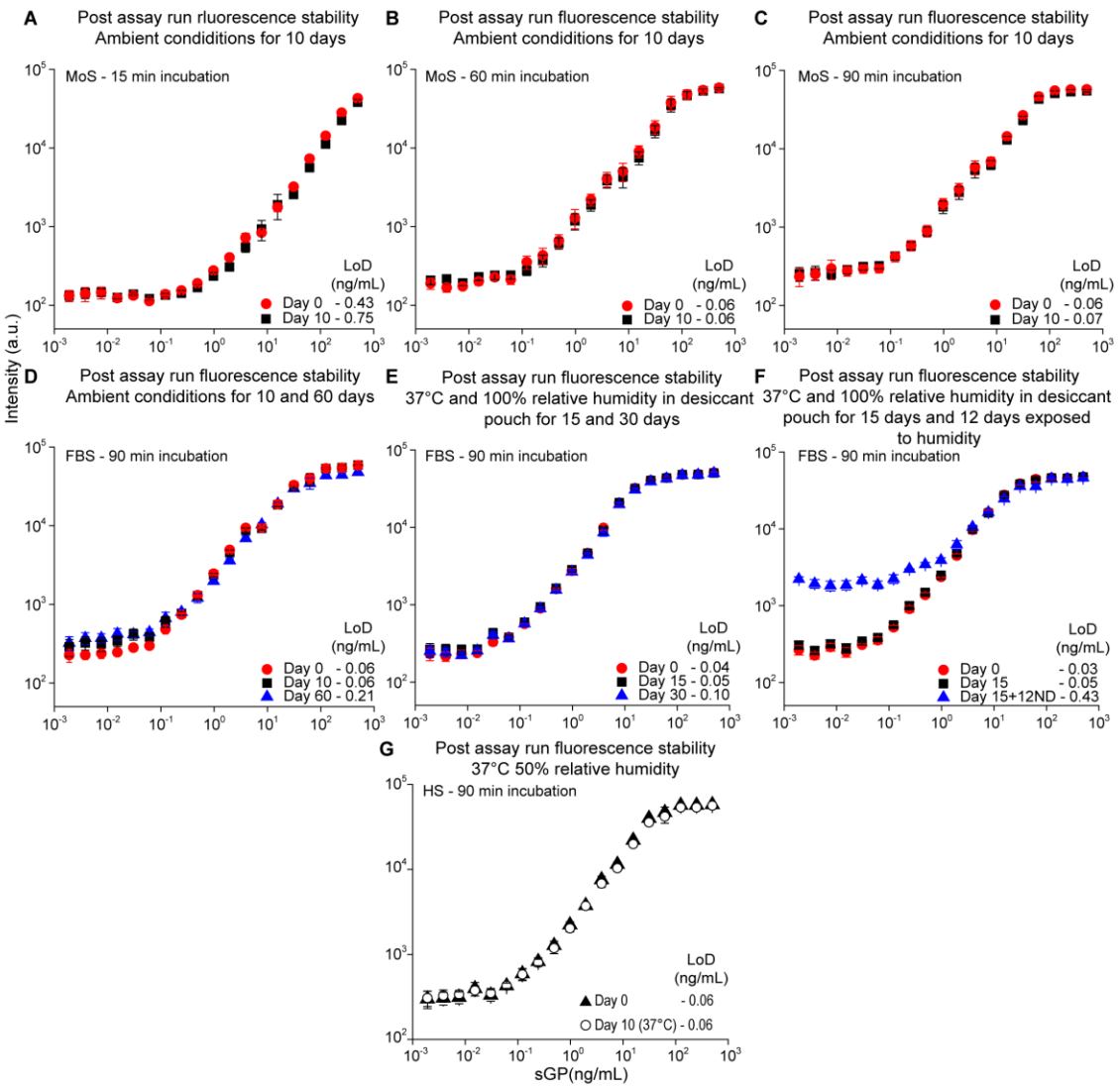
LoB (ng/mL)	LoD (ng/mL)	DR (log10)	UR (log10)
0.02	0.05	5.10	>6.30

iii. Thermal stability

Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 30 at 37°C LoB (ng/mL)	Day 30 at 37°C LoD (ng/mL)	Day 30 at 37°C DR (log10)	Day 60 at 37°C LoB (ng/mL)	Day 60 at 37°C LoD (ng/mL)	Day 60 at 37°C DR (log10)
0.02	0.04	4.12	0.02	0.04	4.06	0.04	0.08	3.77

Fig. S10. EBOV D4 assay performance. Dose-response curves using A1F3-1 as cAb and C2BA5-2 as dAb for different incubation times. Each data point represents the mean ± SEM of *N* = 4 independent D4 assays. Dashed lines show a 5-parameter logistic fit used to calculate LoB, LoD, and DR. **(A)** D4 assay conducted on sGP-spiked calf serum. Dose-response curves and LoDs are shown for incubation times of 15, 30, 60, and 90 min. D4 assay conducted on sGP-

spiked **(B)** pooled human serum**, (C)** pooled human serum without blocking reagent (TruBlock) on test devices, **(D)** rhesus macaque serum, **(E)** D4 assay figures-of-merit (FOMs; LoB, LoD, DR) **(i)** D4 assay results from different sample matrices for different incubation times. **(ii)** 8-log dose-response curve using sGP-spiked calf-serum and a 90 min incubation. **(iii)** Thermal stability of tests stored in aluminum pouches with desiccant at 37°C for 30 and 60 days. UR, useful range; NB, no blocking reagent.



H **D4 assay fluorescence output stability**

i. Macaque serum (ambient conditions)

Incubation (minutes)	Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 10 LoB (ng/mL)	Day 10 LoD (ng/mL)	Day 10 DR (log10)
15	0.17	0.43	3.07	0.17	0.75	2.82
60	0.004	0.06	3.91	0.004	0.06	3.93
90	<0.001	0.06	3.94	<0.001	0.07	3.83

ii. FBS (ambient conditions) 90 minutes incubation

Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 10 LoB (ng/mL)	Day 10 LoD (ng/mL)	Day 10 DR (log10)	Day 60 LoB (ng/mL)	Day 60 LoD (ng/mL)	Day 60 DR (log10)
0.02	0.06	3.91	0.006	0.06	3.89	0.12	0.21	3.38

iii. Human serum (37°C 50% rel. humid.) 90 minutes incubation

Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 10 LoB (ng/mL)	Day 10 LoD (ng/mL)	Day 10 DR (log10)
0.01	0.06	3.91	0.01	0.06	3.89

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

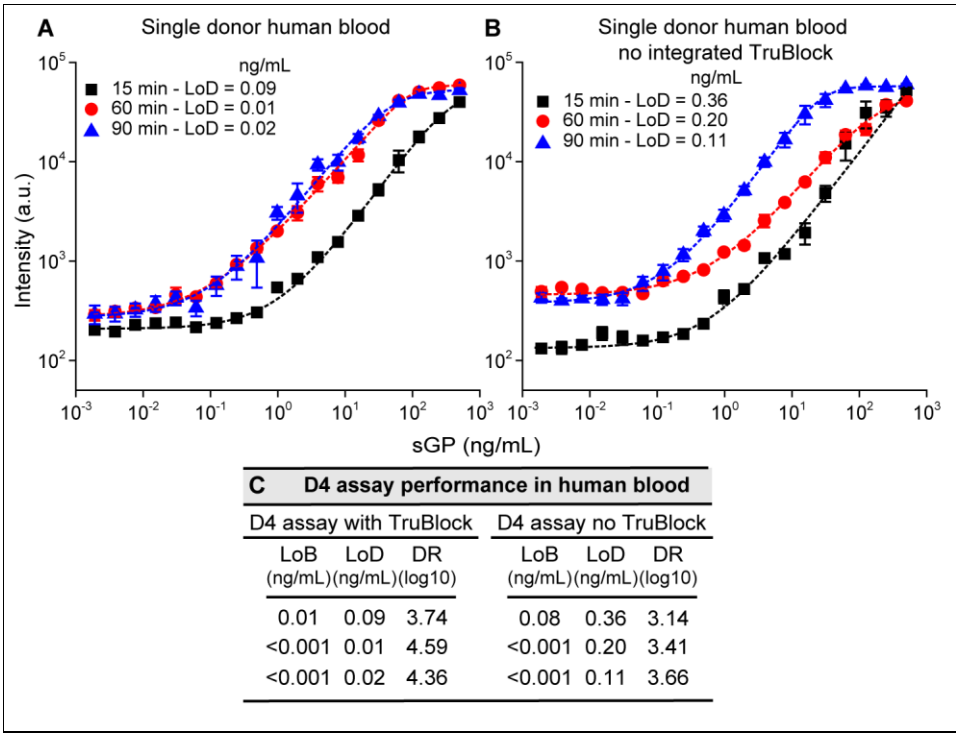
Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 15 LoB (ng/mL)	Day 15 LoD (ng/mL)	Day 15 DR (log10)	Day 30 LoB (ng/mL)	Day 30 LoD (ng/mL)	Day 30 DR (log10)
<0.001	0.04	4.13	0.04	0.05	4.01	0.09	0.10	3.70

v) FBS (Dessicant Pouch - 37°C followed by high relative humidity exposure at 37°C) 90 minutes incubation

Day 0 LoB (ng/mL)	Day 0 LoD (ng/mL)	Day 0 DR (log10)	Day 15 LoB (ng/mL)	Day 15 LoD (ng/mL)	Day 15 DR (log10)	Day 15 + 12ND LoB (ng/mL)	Day 15 + 12ND LoD (ng/mL)	Day 15 + 12ND DR (log10)
0.02	0.03	4.17	0.03	0.05	4.05	0.19	0.43	3.07

Fig. S11. Post-assay fluorescence stability. (A-C) D4 assay dose-response curves obtained using sGP-spiked rhesus monkey serum and incubation times of 15 min **(A)**, 60 min **(B)**, and 90

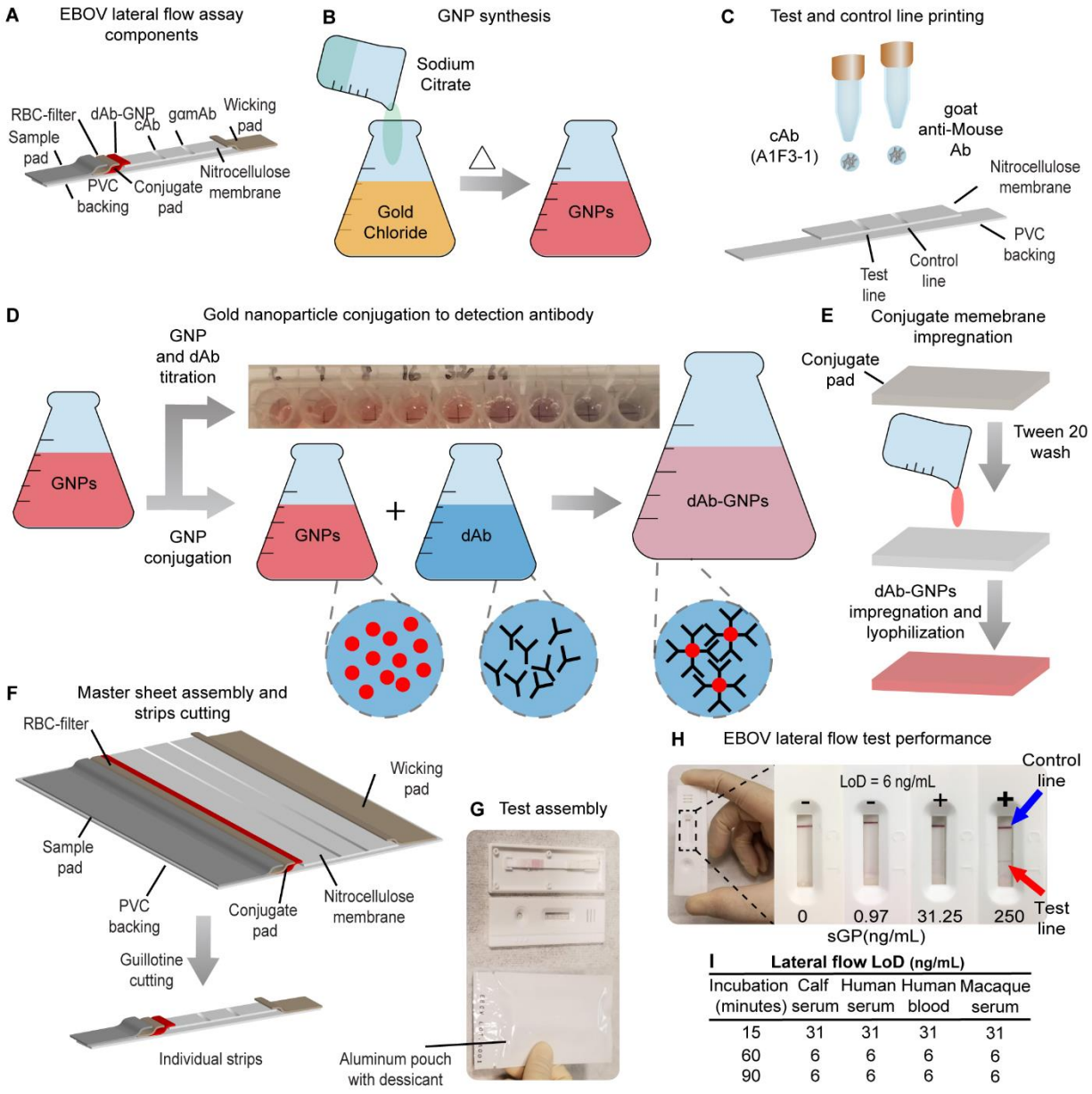
min **(C)**, after 0 days (red) and 10 days (black) of exposure to ambient temperature and relative humidity. **(D)** D4 assay dose-response curves using sGP-spiked FBS and a 90 min incubation after 0 days (red), 10 days (black), and 60 days (green) of exposure to ambient temperature and relative humidity. **(E)** D4 assay dose-response curves using sGP-spiked FBS and a 90 min incubation after 0 days (red), 15 days (black), and 30 days (green) of storage at 37°C in a sealed pouch with desiccant. **(F)** D4 assay dose-response curves using sGP-spiked FBS and a 90 min incubation after 0 days (red) and 15 days (black) of storage at 37°C in a sealed pouch with desiccant, and after 12 days of exposure to 37°C and 100% relative humidity following removal from dry pouch (green). **(G**) D4 assay dose-response curves using sGP-spiked pooled human serum and a 90 min incubation after 0 days (black) and 10 days (white) of exposure to 37°C and 50% relative humidity. Data were fit using a 5-parameter logistic fit (omitted for better data visualization) to calculate LoDs. **(H)** D4 assay figures-of-merit (FOMs; LoB, LoD, DR) for fluorescence output stability experiments. **(i)** FOMs using sGP-spiked rhesus monkey serum and incubation times of 15, 60, and 90 min after 0 and 10 days of exposure to ambient temperature and humidity. **(ii)** D4 assay FOMs using sGP-spiked calf serum and a 90-min incubation after 0, 10, and 60 days of exposure to ambient temperature and humidity. **(iii)** D4 assay FOMs using sGP-spiked pooled human serum and a 90 min incubation after 0 and 10 days of exposure to 37°C and 50% relative humidity. **(iv)** D4 assay FOMs using sGP-spiked FBS and a 90 min incubation after 0, 15, and 30 days of storage at 37°C in a sealed pouch with desiccant. (**v**) D4 assay FOMs using sGP-spiked FBS and a 90 min incubation after 0 and 15 days of storage at 37°C in a sealed pouch with desiccant, followed by 12 days of exposure to 37°C and 100% relative humidity. FOMs, figures of merit; LoB, limit-of-blank; LoD, limit-of-detection; DR, dynamic range. Each data point represents the mean ± SEM of $N = 4$ independent D4 assays



C D4 assay performance in human blood

D4 assay with TruBlock			D4 assay no TruBlock		
LoB (ng/mL)	LoD (ng/mL)	DR (log10)	LoB (ng/mL)	LoD (ng/mL)	DR (log10)
0.01	0.09	3.74	0.08	0.36	3.14
<0.001	0.01	4.59	<0.001	0.20	3.41
<0.001	0.02	4.36	<0.001	0.11	3.66

Fig. S12. EBOV D4 assay performance in human blood. Dose-response curves using A1F3-1 as cAb and C2BA5-2 as dAb for different incubation times. Each data point represents the mean ± SEM of N = 4 independent D4 assays. Dashed lines show a 5-parameter logistic fit used to calculate LoB, LoD, and DR. Dose-response curves and LoDs are shown for incubation times of 15, 60, and 90 min in single donor human whole blood, using D4 tests with Trublock **(A)** and without **(B)**. **(C)** D4 assay figures-of-merit (FOMs; LoB, LoD, DR) for D4 assay with and without Trublock in human blood. FOMs, figures of merit; LoB, limit-of-blank; LoD, limit-of-detection; DR, dynamic range; NB, no blocking reagent



Incubation (minutes)	Calf serum	Human serum	Human blood	Macaque serum
15	31	31	31	31
60	6	6	6	6
90	6	6	6	6

Fig. S13. EBOV sGP lateral flow assay fabrication. (A) Illustration of LFA 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) in test-line region, goat anti-mouse Ab (gamAb) in control-line region, and cellulose-based wicking pad. **(B)** Citrate reduction-based 15 nm diameter gold nanoparticle synthesis. **(C)** Test and control line printing on nitrocellulose membrane with non-contact dispenser. **(D)** Gold

nanoparticle titration to determine optimal dAb titers for GNP stabilization. **(E)** Conjugate membrane impregnation in which GNP-dAb are deposited and freeze-dried on fiberglass. **(F)** Master sheet assembly and strip cutting. Impregnated nitrocellulose membrane is assembled on PVC backing sheet followed by overlapping conjugate and sample pads. The wicking pad is assembled, and individual 4-mm strips are cut using an automated guillotine cutter. **(G)** Test assembly images. Individually cut assay strips are placed in a plastic cassette and into an aluminum pouch with desiccant. Product name and lot numbers are printed on the pouch during sealing. **(H)** EBOV sGP LFA performance. Assay runs with different concentrations of sGP spiked into human serum. 31.25 ng/mL of sGP yields a faint test line; at 250 ng/mL, a positive result is more evident. **(I)** Performance of LFA assays in human blood, calf serum, human serum, and Rhesus monkey serum. The presence of a test line was evaluated for 15, 60, and 90 min incubation at different sGP concentrations to determine the LoD. GNP, gold nanoparticles; RBC, red blood cell.

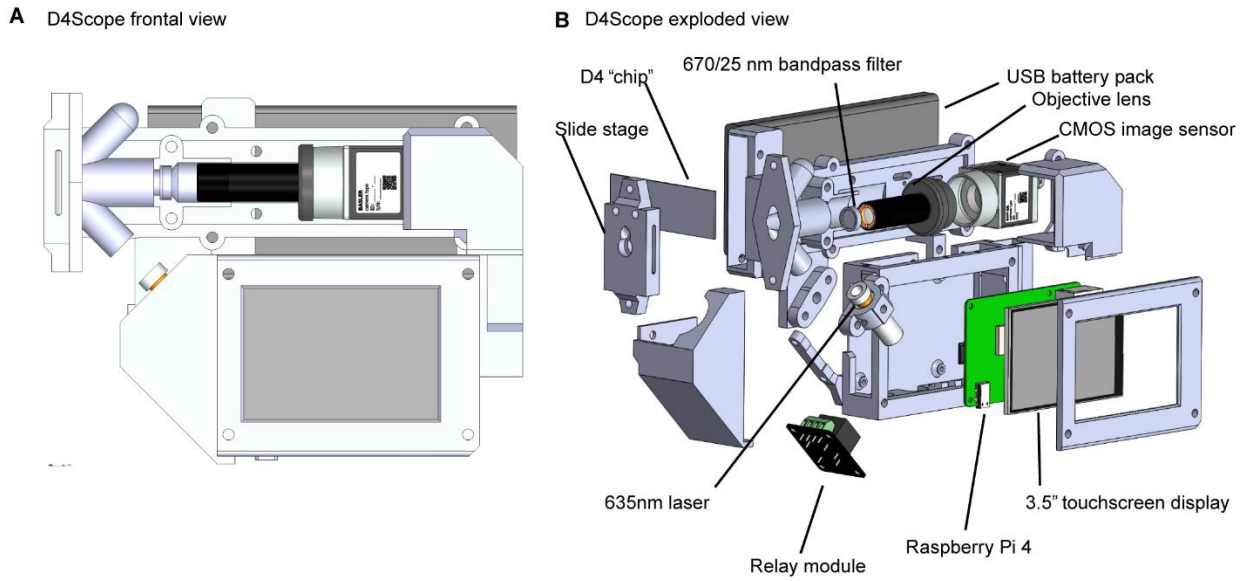


Fig. S14. D4Scope design. (A) Frontal view of D4Scope depicting 3.5-inch touchscreen display and complementary metal oxide semiconductor (CMOS) image sensor. **(B)** Exploded view of D4Scope depicting all the off-the-shelf components used to assemble the D4Scope: USB battery pack, CMOS image sensor, relay module, 3.5-inch touchscreen display, 635 nm laser in a custom-designed 3D-printed body.

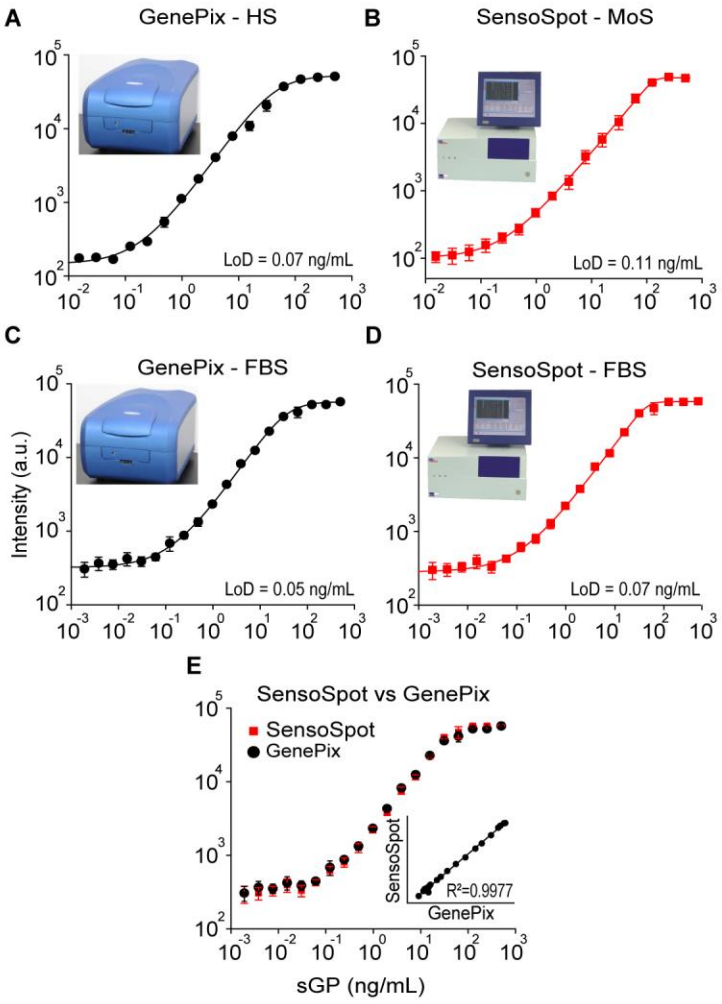


Fig. S15. Comparison of D4Scope, GenePix, and SensoSpot performance. (A) Dose-response curves of sGP in HS with a 90 min incubation using GenePix. **(B)** Dose-response curves of sGP in MoS with a 90 min incubation using SensoSpot at Galveston National Laboratory (BSL-4) by a technician following a 30 min training session. **(C-E)** Dose-response curves of sGP in FBS with a 90 min incubation using GenePix and SensoSpot scanners. Panel E inset: correlation of fluorescence readout between SensoSpot and GenePix. Each data point represents the mean ± SEM of $N = 4$ independent D4 assays.

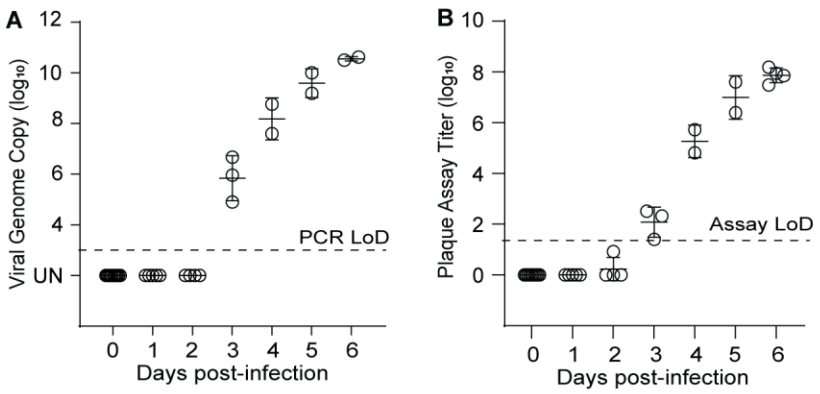


Fig. S16. RT-PCR and plaque assay. Adapted from Versteeg et. al. (*44*). **(A)** Viral genome copies measured using RT-PCR with primers targeting VP30 for each time-point. Each data point is the mean of $N = 2$ technical repeats per sample. Mean ± SD deviation for viral genome copies is indicated for each time-point. **(B)** Infectious virus quantified by plaque assay using Vero cells. Each data point is the mean of $N = 2$ technical repeats per sample. Mean ± SD for PFU is indicated for each time-point. A statistically significant difference between the PCR and PFU/mL readouts was observed starting on Day 3 ($P<0.0001$; one-way ANOVA followed by Tukey's post-hoc test).

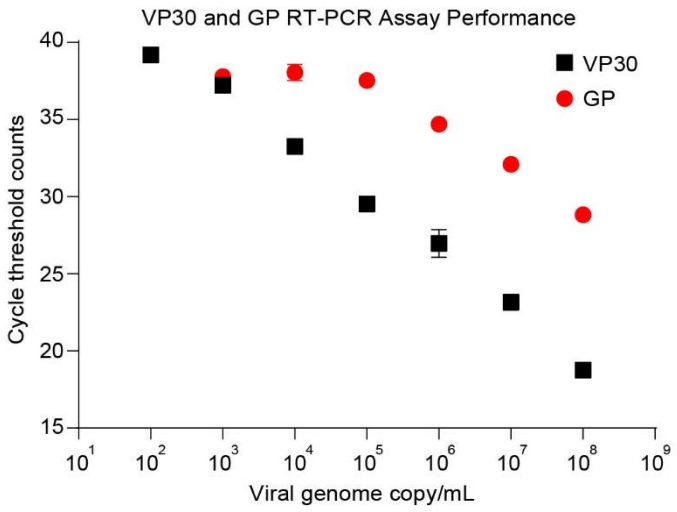


Fig. S17. VP30- and GP-targeting RT-PCR. Standard curves of cycle threshold (Ct) counts and viral genome copies per mL are shown for the RT-PCR using primers targeting EBOV GP and VP30 genes. Ten-fold serial dilutions are linear over approximetly seven logs for the assay targeting VP30 gene with a LoD ranging between 100 and 1000 copies per mL and four logs for the assay targeting GP gene with a LoD of ~10,000 RNA copies/mL of sample. Data represents mean ± SEM of at least N = 2 technical replicates per data-point and N = 6 to12 for the points in the linear range of the assays. Linear fits were performed with GraphPad Prism. VP30 targeting assay presented Slope = -3.394; Error = 0.09430; R^2 = 0.9693 and Efficiency = 1.97 while the GP targeting assay presented Slope = -2.875; Error = 0.1195; R^2 = 0.9324 and Efficiency = 2.23 in their linear regimes. Efficiency (E) is deffined as $E=10^{-1/Slope}$

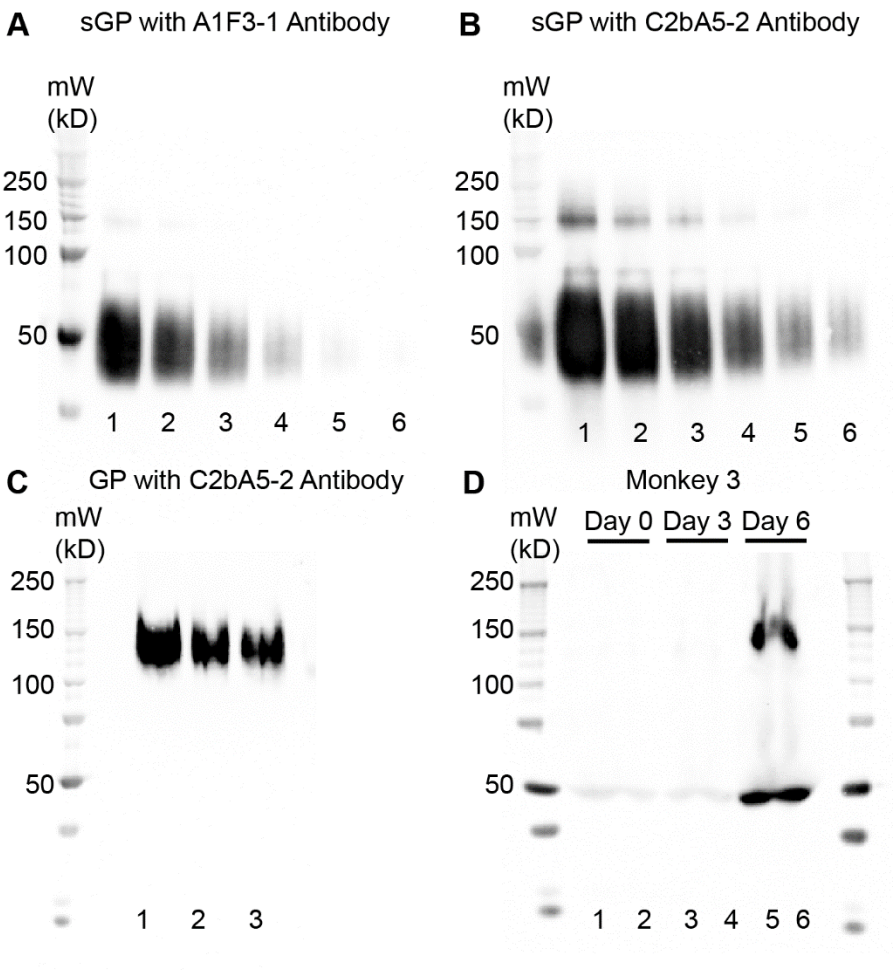


Fig. S18. Western blot of MoS samples. (A-B) Western blot results for EBOV sGP using A1F3-1**(A)** and C2bA5-2**(B)** antibody and visualized by chemiluminescence. sGP was diluted in RIPA buffer, treated with equal volume of 4 X Laemmli sample buffer and heated to 95°C for 10 min. Lanes 1-6: Signal intensity for (1) 2.36 µg, (2) 1.18 µg, (3) 0.59 µg, (4) 0.30 µg, (5) 0.15 µg, (6) 0.07 µg of sGP. **(C)** Western blot results for EBOV GP using C2bA5-2 antibody and visualized by chemiluminescence. GP was diluted in RIPA buffer, treated with equal volume of 4 X Laemmli sample buffer and heated to 95°C for 10 min. Lanes 1-3: Signal intensity for (1) 0.25 µg, (2) 0.125 µg, (3) 0.063 µg. **(D)** Western blot results for serum from EBOV challenged rhesus monkey samples using C2bA5-2 and goat anti-mouse conjugated to horseradish peroxidase and Precision Protein StrepTactin-HRP Conjugate antibody as developing reagents.

Samples collected on days 0, 3 and 6. Samples were heat inactivated (95°C for 10 min) with an equal volume of 4X Laemmli sample buffer and equal amounts were loaded in each well. Lanes (1-2) Day 0, (3-4) Day 3, (5-6) Day 6. Results indicate that sGP and GP are present in high concentration at later stages of infection, but lanes Days 0 and 3 indicate low level of sGP, and no visible level of GP. The faint band referent to sGP seen on days 0 can be related to high concentration of sGP in the sample from Day 6, which can cause ghost bands, Day 0 sample was collected before viral challenge. MW, molecular weight; kD, kilodalton.

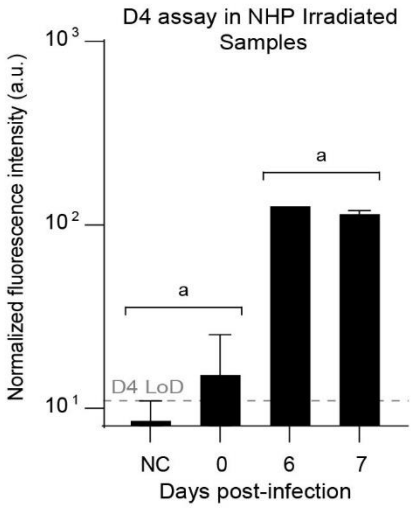


Fig. S19. Irradiated samples from EBOV-challenged nonhuman primates. 3 healthy filovirus-negative cynomolgus macaques were challenged IM with 1,000 PFU of EBOV Makona strain. Serum samples were collected at Days 0, 6 and 7 were inactivated by gamma irradiation (~5 mrad) according to protocols approved by local Institutional Biosafety Committee (IBC). Following inactivation, these samples were tested with the D4 assay to assess if gamma irradiation could compromise D4 assay performance. Normalized fluorescence intensity values are reported for each time point. Each data point shows the mean ± SD of all samples tested per time-point. Non-irradiated pooled human serum was used as a negative control (NC). There was no statistically significant difference between the fluorescence values of NC and Day 0. A statistically significant difference between fluorescence values of NC and Day 0 versus those collected at Days 6 and 7 was seen (P<0.0001; one-way ANOVA followed by Tukey's post-hoc test); values labeled with the same letter are significantly different. Samples from Day 0 were PCR negative and those from Day 6 and 7 highly positive.

Table S1. D4Scope cost estimate. Cost estimate is based on small numbers of bought parts. Cost of 3D printed parts is estimated based on amount of material used to fabricate each component of the D4Scope body.

D4Scope Single Unit Cost	
Item	**Price (US$)**
Basler Ace Camera	240.00
Opto Engineering MC100X Lens	349.00
Semrock F01-676/37-25 25 mm	225.00
Red laser (635 nm) Sharp 185 mW	93.00
USB micro B 3.0 camera cable	13.88
3D printed parts	10.99
Raspberry Pi 4B	35.00
Total	966.87

Table S2. Summary of nonhuman primate model results. PCR and PFU results are $\log_{10}$ of viral genome copies/mL. Normalized D4 assay fluorescence levels are reported in triplicate; sGP levels are calculated using the dose-response curve in **Fig. 4E**. "-" indicates assay not performed. LoD, limit-of-detection; PFU, plaque forming unit.

Intramuscular Challenge								
Macaque ID	Day	Log10 copies/mL		Log10 PFU/mL	D4Scope Normalized Fluorescence			sGP Conc. (ng/mL)
M1	0	<LOD	<LOD	0	10.80	11.17	10.38	<LOD
	1	<LOD	<LOD	0	11.09	10.61	10.78	<LOD
	2	<LOD	<LOD	0	12.97	11.81	11.03	0.25
M2	0	<LOD	<LOD	0	11.01	10.65	9.01	<LOD
	1	<LOD	<LOD	0	11.18	8.41	8.41	<LOD
	2	<LOD	<LOD	0	11.17	8.05	7.45	<LOD
M3	0	<LOD	<LOD	0	-	9.22	10.72	<LOD
	3	6.00	5.92	2.52	43.40	49.24	70.85	8.07
	4	8.79	8.74	5.72	340.18	229.92	416.54	>500
M4	0	<LOD	<LOD	0	9.00	10.34	10.65	<LOD
	3	4.92	4.88	1.40	12.33	-	19.70	0.90
	6	10.51	10.49	7.86	96.70	112.18	161.17	23.68
M5	0	<LOD	<LOD	0	8.52	11.10	9.86	<LOD
	3	6.67	6.66	2.32	155.34	130.42	140.87	28.98
	6	10.59	10.65	7.48	69.43	79.63	42.22	9.95
M6	0	<LOD	<LOD	0	7.90	9.26	9.75	<LOD
	1	<LOD	<LOD	0	9.38	12.99	11.51	<LOD
	4	7.60	7.61	4.81	366.80	274.74	345.54	>500
M7	0	<LOD	<LOD	0	10.11	11.04	11.32	<LOD
	1	<LOD	<LOD	0	14.81	11.74	12.40	0.41
	2	<LOD	<LOD	0	10.42	13.47	13.1	0.31
M8	0	<LOD	<LOD	0	7.74	7.92	10.99	<LOD
	1	<LOD	<LOD	0	9.29	10.64	10.89	<LOD
	2	<LOD	<LOD	0.92	12.42	10.46	15.69	0.41
M9	0	<LOD	<LOD	0	11.59	10.59	6.93	<LOD
	5	9.19	9.19	7.6	200.47	140.54	101.34	30.59
	6	-	-	7.94	45.63	54.89	40.96	6.62
M10	0	<LOD	<LOD	0	12.68	11.99	7.90	<LOD
	5	9.06	9.10	6.39	247.42	207.01	242.99	83.51
	6	-	-	8.18	136.56	106.66	115.20	22.67

Table S3. Summary of irradiated samples from EBOV-challenged nonhuman primates. 3 healthy filovirus-negative cynomolgus macaques were challenged intramuscularly with 1,000 PFU of EBOV Makona strain. Blood and serum samples were collected at the indicated time-points. Blood samples were used to determine viral genome copies measured using RT-PCR with primers targeting VP30. Serum samples were inactivated by gamma irradiation (~5 mrad) and tested for anti-GP antibodies by ELISA, to ensure that no anti-GP antibody was present in these samples, as these animals were also used in other undisclosed studies at UTMB. Irradiated serum samples were then tested in the D4 assay. Fluorescence output results were quantified using the SensoSpot tabletop scanner and sGP concentration determined using the dose-response curve presented in **fig. S15B.** A non-irradiated pooled human serum sample was used as negative control. "-" indicates assay not performed.

Macaque ID	Day	Log10 copies/mL	SensoSpot Normalized Fluorescence	sGP Conc. (ng/mL)	ELISA (EBOV GP Abs)
NC (non-irradiated)	-	-	10.00 10.16 6.46	<LOD	-
IrMA	0	<LOD	26.70	3.21	Undetected
	7	12.5	118.81	22.04	Undetected
IrMB	0	<LOD	9.94	<LOD	Undetected
	7	12.0	113.66	20.98	Undetected
IrMC	0	<LOD	10.67	<LOD	Undetected
	6	12.0	130.71	24.50	Undetected

Data file S1. Raw data. (Excel file)