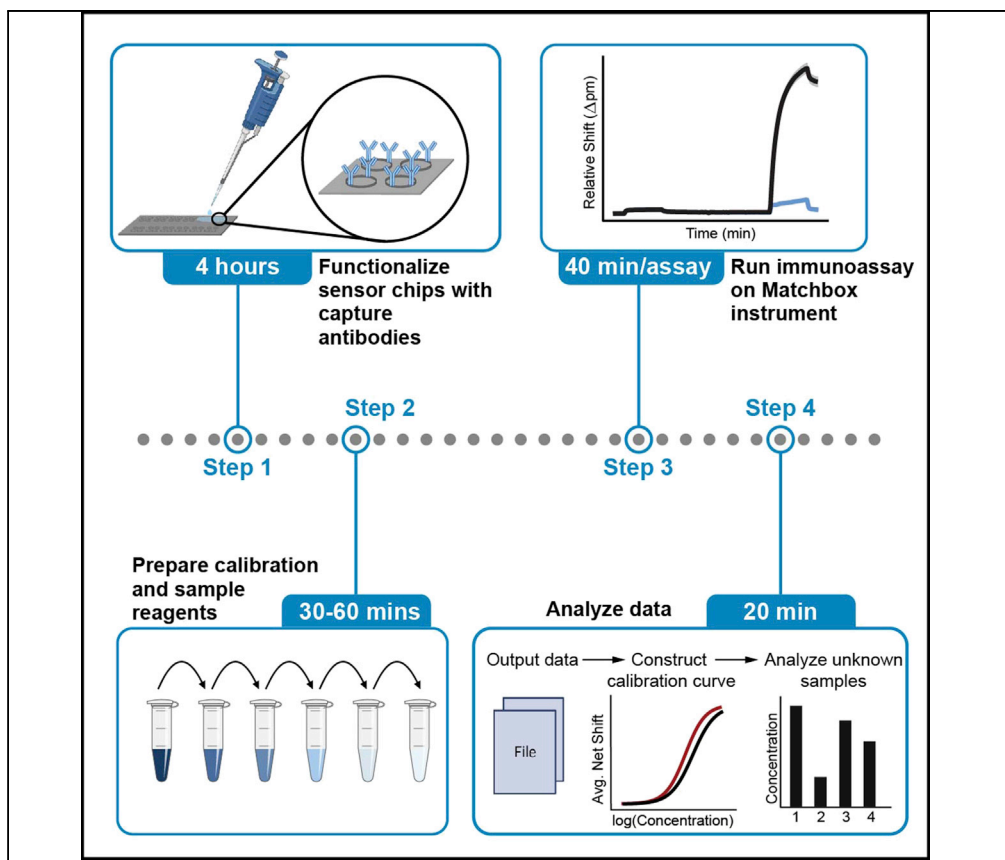


## Protocol

# Detection of biomarkers for filoviral infection with a silicon photonic resonator platform



This protocol describes the use of silicon photonic microring resonator sensors for detection of Ebola virus (EBOV) and Sudan virus (SUDV) soluble glycoprotein (sGP). This protocol encompasses biosensor functionalization of silicon microring resonator chips, detection of protein biomarkers in sera, preparing calibration standards for analytical validation, and quantification of the results from these experiments. This protocol is readily adaptable toward other analytes, including cytokines, chemokines, nucleic acids, and viruses.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Optimized protocol for detection of viral biomarkers using microring resonator sensors

Functionalization of biosensor surface with specific capture antibodies

Construction of assay calibration standards and non-human primate serum samples

Data processing for conversion of data output to analyte concentration

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## Protocol

## Detection of biomarkers for filoviral infection with a silicon photonic resonator platform

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## SUMMARY

**This protocol describes the use of silicon photonic microring resonator sensors for detection of Ebola virus (EBOV) and Sudan virus (SUDV) soluble glycoprotein (sGP). This protocol encompasses biosensor functionalization of silicon microring resonator chips, detection of protein biomarkers in sera, preparing calibration standards for analytical validation, and quantification of the results from these experiments. This protocol is readily adaptable toward other analytes, including cytokines, chemokines, nucleic acids, and viruses.**

**For complete details on the use and execution of this protocol, please refer to Qavi et al. (2022).**

## BEFORE YOU BEGIN

The protocol below describes the specific steps for detection of EBOV sGP and SUDV sGP using the microring resonator sensing platform. However, we have used this protocol for detection of varying cytokines (Kindt et al., 2013), chemokines (Valera et al., 2016), nucleic acids (Qavi et al., 2011), and viruses (McClellan et al., 2012). Additionally, we have used this protocol for detection of cytokines and chemokines in clinical samples for precision medicine applications (Robison et al., 2019, 2021).

## Institutional permissions

All animal studies were performed under approval of the local IACUC committees. All work was performed in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals. The USARMIID is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council.



### Non-human primate samples

The collection of serum samples must be approved through appropriate Institutional Review Boards prior to any studies and be collected in accordance with local laws and regulations.

△ **CRITICAL:** Patient serum and plasma samples carry risk of pathogens, not limited to hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV). Therefore, proper PPE must be utilized when handling any samples (Twitchell, 2003).

### Antibody desalting and dilution

⌚ **Timing:** 30 min

1. Desalt the stock capture antibody solutions to remove azide from the formulation. The [Buffer Exchange Procedure](#) in the package insert from Thermo Scientific was followed.
  - a. Remove columns' bottom closure and loosen cap.
  - b. Place column in 1.5 mL collection tube and centrifuge at 1,500 g for 1 min. Discard filtrate.
  - c. Mark side of column where resin is slanted upward, place column in same orientation for each following spin.
  - d. Add 300  $\mu$ L of 1  $\times$  PBS on top of resin bed, place back into 1.5 mL collection tube and centrifuge at 1,500 g for 1 min. Discard filtrate. Repeat for a total of 3 resin washes.
  - e. Place column in new collection tube, pipette 50–100  $\mu$ L of antibody on top of resin bed.
  - f. Centrifuge at 1,500 g for 2 min and collect filtrate.
2. Measure the absorbance of the resulting filtrate from step 1 at 280 nm using the Nanodrop1000 Spectrophotometer (Thermo Fisher Scientific) or equivalent.
  - a. In the software panel, select Protein A280.
  - b. Initialize the instrument by pipetting 1  $\mu$ L of ultrapure water onto the measurement pedestal and initialize the software.
  - c. Clean the pedestal with Deionized (DI) water and blank by pipetting 1  $\mu$ L of 1  $\times$  PBS onto the measurement pedestal. Click 'Blank' on the software.
  - d. Clean the pedestal with DI water and pipette 1  $\mu$ L of antibody sample onto the pedestal. Click 'Measure Sample' on the software.
  - e. Record the mg/mL output. Repeat step 2d twice more and then calculate the average mg/mL.
  - f. Dilute the filtered antibody solution to 0.5 mg/mL using 1  $\times$  PBS, aliquot into 5  $\mu$ L aliquots and store at  $-80^{\circ}\text{C}$  until use.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-EBOV capture Ab, desalted, stored at 0.5 mg/mL (1:2)	Integrated BioTherapeutics	Cat#: 0365-001
Anti-SUDV capture Ab, stored at 0.5 mg/mL (1:2)	Integrated BioTherapeutics	Cat#: 0302-030
Biotinylated Anti-EBOV/SUDV pan Ab, stored at 0.5 mg/mL (1:277)	Integrated BioTherapeutics	N/A
M $\mu$ IgG control Ab, 5 mg/mL (1:23)	Thermo Fisher Scientific	Cat #: 0031903
<b>Biological samples</b>		
NHP serum	N/A	N/A
Pooled healthy serum	Innovative Research, Novi, MI	Cat #: ISER50ML
<b>Chemicals, peptides, and recombinant proteins</b>		
Recombinant EBOV Soluble GP (sGP)	Integrated BioTherapeutics	Cat#: 0565-001
Recombinant SUDV Soluble GP (sGP)	Integrated BioTherapeutics	Cat#: 0570-001
Acetone (7.9 mL)	Fisher Chemical	Cat#: A184
Isopropanol (2 mL)	Fisher Chemical	Cat#: A4154
3-aminopropyltriethoxysilane (APTES, 100 $\mu$ L)	Sigma Aldrich	Cat#: 440140

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bis(sulfosuccinimidyl)suberate (BS3, 2 mg)	Thermo Fisher Scientific	Cat#: A39266
Acetic acid (2 mM)	Fisher Chemical	Cat#: A38-212
Glycerol (50% solution)	Sigma-Aldrich	Cat#: G5516
DryCoat Assay Stabilizer	Virusys Corporation	Cat#: AG066-1
Starting Block Buffer	Thermo Fisher Scientific	Cat#: 37578
Streptavidin horse radish peroxidase (SAHRP)	Thermo Fisher Scientific	Cat#: 21130
4-chloro-1-naphthol (4CN)	Thermo Fisher Scientific	Cat#: 34012
<b>Software and algorithms</b>		
Genalyte Matchbox User Interface	Genalyte, Inc.	MavII Host 8.2
RStudio: Integrated Development Environment for R	RStudio, Inc.	Version 1.2.5001
Nanodrop1000 User Interface	Thermo Scientific	Version 3.8.1
<b>Other</b>		
Matchbox Instrument, Maverick Detection System	Genalyte Inc.	N/A
Unstripped Silicon Photonic Chips	Genalyte, Inc.	N/A
Chip Carriers with Back Door Adhesive	Genalyte Inc.	N/A
Zeba Sip Desalting Columns, 7K MWCO	Thermo Scientific	Cat#: 89882
Vortexer	Fisher Scientific	Cat#: 12-812
Microscope	Leica	Cat#: EZ4W
The Belly Dancer Orbital Shaker	IBI Scientific	Model#: BDRAA115S
Microcentrifuge tubes (0.6 mL)	Fisher Scientific	Cat#: 02-682-000
Microcentrifuge tubes (1.5 mL)	Fisher Scientific	Cat#: 02-682-002
Microcentrifuge tubes (5 mL)	Fisher Scientific	Cat#: 14-282-300
DURX 670 cleanroom wipe	Berkshire	Cat#: DR670.0909.20
24 well plate with lid	Thermo Scientific	Cat#: 142485
96 well V-bottom plate	Corning	Cat#: 3357
20 mL Glass Screw-Thread Scintillation Vials	Fisher Scientific	Cat#: 03-340-4L
Monoject 1 mL Tuberculin Syringe (Regular Tip)	Fisher Scientific	Cat#: 22-257-135
BD PrecisionGlide Needle, 26G × 1/2	Fisher Scientific	Cat#: 14-826-15
Nanodrop1000 Spectrophotometer	Thermo Scientific	Cat#: ND-1000(US\CAN)

**Note:** Any version of RStudio or other data processing software, such as excel, will work for this purpose. Any nanodrop or spectrophotometer that measures samples at 280 nm will work for this purpose.

## MATERIALS AND EQUIPMENT

### 1 × PBS

Reagent	Final concentration	Amount
Phosphate Buffered Saline (solid)	137 mM NaCl, 10 mM phosphate	9.6 g
Ultrapure water	N/A	1,000 mL
<b>Total</b>	<b>N/A</b>	<b>1,000 mL</b>

Filter through 0.2 μm filter and store at Store at 18°C–22°C for no longer than 12 months.

**Alternatives:** Solid sodium chloride and sodium phosphate can be substituted for phosphate buffered saline with final concentrations matched to above.

### 1 × PBS-BSA

Reagent	Final concentration	Amount
Phosphate Buffered Saline (solid)	137 mM NaCl, 10 mM phosphate	9.6 g
Bovine Serum Albumin	0.5%	5 g
Ultrapure water	N/A	1,000 mL
<b>Total</b>	<b>N/A</b>	<b>1,000 mL</b>

Filter through 0.2 μm filter and store at 4°C for up to 6 months.

**Alternatives:** Solid sodium chloride and sodium phosphate can be substituted for phosphate buffered saline with final concentrations matched to above.

50% Glycerol		
Reagent	Final concentration	Amount
Glycerol (liquid)	50%	500 $\mu$ L
Ultrapure water	N/A	500 $\mu$ L
<b>Total</b>	<b>N/A</b>	<b>1 mL</b>

Store at 18°C–22°C for no longer than 12 months.

## STEP-BY-STEP METHOD DETAILS

### Functionalization of sensor chip

⌚ Timing: 3–4 h

This section describes the method to functionalize the silicon photonic microring sensor chips through silanization of the chip surface and use of a homo-bifunctional linker molecule to covalently attach the antibodies to the microring sensors. This step requires about 1–2 h of active work and two 1 h incubations.

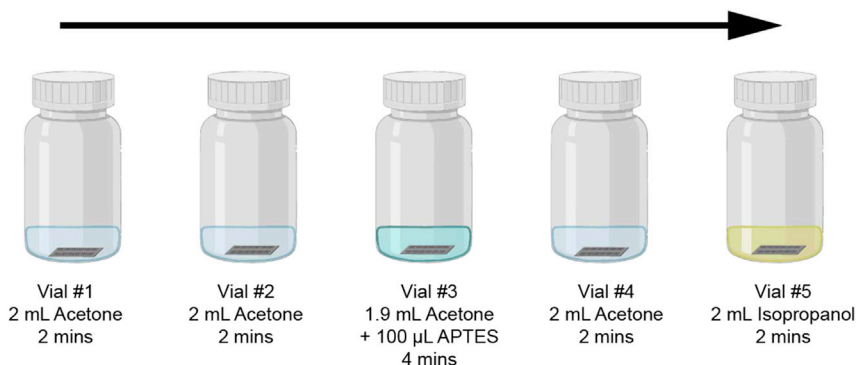
#### 1. Capture Antibody Preparation.

This should be completed prior to the following steps to mitigate time restraints later in the protocol.

- In a 0.6 mL microcentrifuge tube, add 4  $\mu$ L of 1 $\times$  PBS, 1  $\mu$ L of 50% glycerol and 5  $\mu$ L of anti-EBOV antibody at 0.5 mg/mL.
- In a second 0.6 mL microcentrifuge tube, add 4  $\mu$ L of 1 $\times$  PBS, 1  $\mu$ L of 50% glycerol and 5  $\mu$ L of anti-SUDV stored at 0.5 mg/mL.
- In a third 0.6 mL microcentrifuge tube, add 25.7  $\mu$ L of 1 $\times$  PBS, 3  $\mu$ L of 50% glycerol and 1.3  $\mu$ L of Ms IgG control antibody stored at 5 mg/mL.
  - The final concentration of capture antibodies is 0.25 mg/mL in 5% glycerol.
- Store the diluted antibodies at 4°C until use. It is best to make fresh the day of spotting.

#### 2. Silanization of Sensor Chip.

- Collect five 20-mL scintillation vials and label them 1–5 (Figure 1).
- Fill with the following:
  - Vials #1, #2, and #4: 2 mL acetone.
  - Vial #3: 1.9 mL acetone.
  - Vial #5: 2 mL isopropanol.
- Using clean tweezers, immerse up to six silicon photonic chips in scintillation vial #1. Secure vial onto orbital shaker and gently agitate for 2 min.
- Using tweezers, transfer the chips to Vial #2. Secure vial onto orbital shaker and gently agitate for 2 min.
- While chips are in Vial #2, prepare 5% 3-Aminopropyltriethoxysilane (APTES) solution.
  - Remove APTES from desiccated storage area.
  - Use a syringe fitted with a needle tip to remove 100  $\mu$ L of APTES from the bottle.
  - Dispense APTES into scintillation vial #3 and recap vial.
  - Return APTES to desiccated storage.
- Transfer the chips to scintillation Vial #3. Secure vial onto orbital shaker and gently agitate for 4 min.
- Transfer the chips to scintillation Vial #4. Secure vial onto orbital shaker and gently agitate for 2 min.

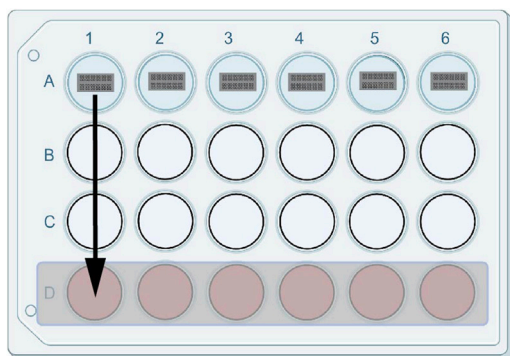


**Figure 1. Sensor chip silanization diagram**

The sensor chips should be moved through each vial after incubating on the orbital shaker for the specified amount of time (step 2). Created with [BioRender.com](https://BioRender.com).

- h. Transfer the chips to scintillation Vial #5. Secure vial onto orbital shaker and gently agitate for 2 min.
  - i. While chips are in Vial #5, fill the top row (A) of 6 wells in the 24-well plate with deionized water ([Figure 2](#)).
  - j. Transfer the chips to the water filled wells, with one chip per well.
  - k. Remove chips from water and place onto a DURX 670 cleanroom wipe.
  - l. Dry surface with N<sub>2</sub> gas line while holding the chip securely with tweezers. Place the dried chips in the second row (B) of wells in the 24 well plate.
  - m. Prepare the BS3 reagent to 5 mM by adding 700 µL acetic acid to a 2 mg no weigh format vial of BS3. Vortex to fully dissolve solid.
    - i. The BS3 solution should be prepared fresh and added to the chip shortly after, as once prepared it is a limited active time (1 h) before hydrolysis of the NHS-ester moieties occur.
  - n. Immediately pipette 20 µL of dissolved BS3 onto the sensor surface. Be sure to fully cover the microring sensor portion of the chip.
  - o. Incubate for 3 min. Briefly dip chips into the water in the first row (A) of the 24 well plate to rinse off BS3 and dry with N<sub>2</sub> gas. Place dried chips into third row (C) of 24-well plate.
  - p. Cut a strip of a DURX 670 cleanroom wipe that is the length and width of the last row (D) of the 24 well plate. Dampen the strip with water and press onto the lid of the 24-well plate over the bottom row. This will create a humidity chamber to house the spotted chips.
3. Antibody Spotting.
- a. Prepare chip spotting station at microscope by collecting the prepared chips, the 0.25 mg/mL antibodies, tweezers, 2.5 µL pipette set from 0.2–0.4 µL, pipette tips, and sensor layout map to annotate ([Figure 3A](#)).
  - b. Place the first chip under the microscope, adjust the settings to 25× magnification and adjust focus on the sensor chip.
  - c. Deposit 0.2–0.4 µL of the first antibody over 2–5 ring clusters in each of the two channels of rings. The exact volume can vary, lower volumes will cover fewer clusters and higher volumes will cover more clusters.
  - d. Repeat with the remaining antibodies moving across the chip.
    - i. Take care to avoid any merging of drops of different antibodies, as this will result in cross reactivity. Troubleshooting [problem 1](#).
    - ii. Using this hand spotting method, it is reasonable to deposit 2–4 antibodies in each channel, with each antibody spanning 2–4 clusters of microrings. For higher plexity, this spotting process would need to be completed through microspotting procedures. However, for this protocol, only three antibodies (anti-EBOV sGP, anti-SUDV sGP and anti-Ms IgG control) were needed in each channel.

For this work, the spotting generally looked like [Figures 3B–3D](#).



**Figure 2. 24-well plate set up during sensor chip functionalization**

Row A contains DI water and houses the sensor chip after silanization (step 2j). The sensor chip moves to row B for BS3 application (step 2l) and to row C after removing the excess BS3 (step 2o). Finally, the sensor chip is housed in the humidity chamber created in row D after the capture antibody has been applied (step 3f). Created with [BioRender.com](https://www.biorender.com/).

- e. Once all antibodies have been spotted, note down which clusters of rings are covered by each antibody. Label this as chip #1. Troubleshooting [problem 2](#).
- f. Carefully transfer the completed chip to the final row (D) of the 24-well plate in the first position. Cover with well plate lid to create humidity chamber.
- g. Repeat steps b-f with remaining chips, placing the next chip in the subsequent spot in row D. It is important to know which spotting map layout corresponds to which chip.
- h. Leave chips at room temperature (18°C–22°C) in the humidity chamber for 1 h.
- i. Pipette 1 mL of starting block into each of the wells. Incubate chips in the starting block at room temperature (18°C–22°C) under the humidity chamber for 1 h.
- j. Remove chips from the 24-well plate and place on the DURX 670 cleanroom wipe. Take care to keep them in the same order. Dry with N<sub>2</sub> gas.
- k. Pipette 20 µL of dry coat solution over the surface of each chip. Gently tap the sides of the chip with the tweezers to wick off excess dry coat onto the DURX 670 cleanroom wipe. There should remain a thin layer of dry coat over the chip to stabilize the antibodies.
- l. Place the chips into a gel pack. Place gel pack in a desiccator in a 4°C refrigerator.

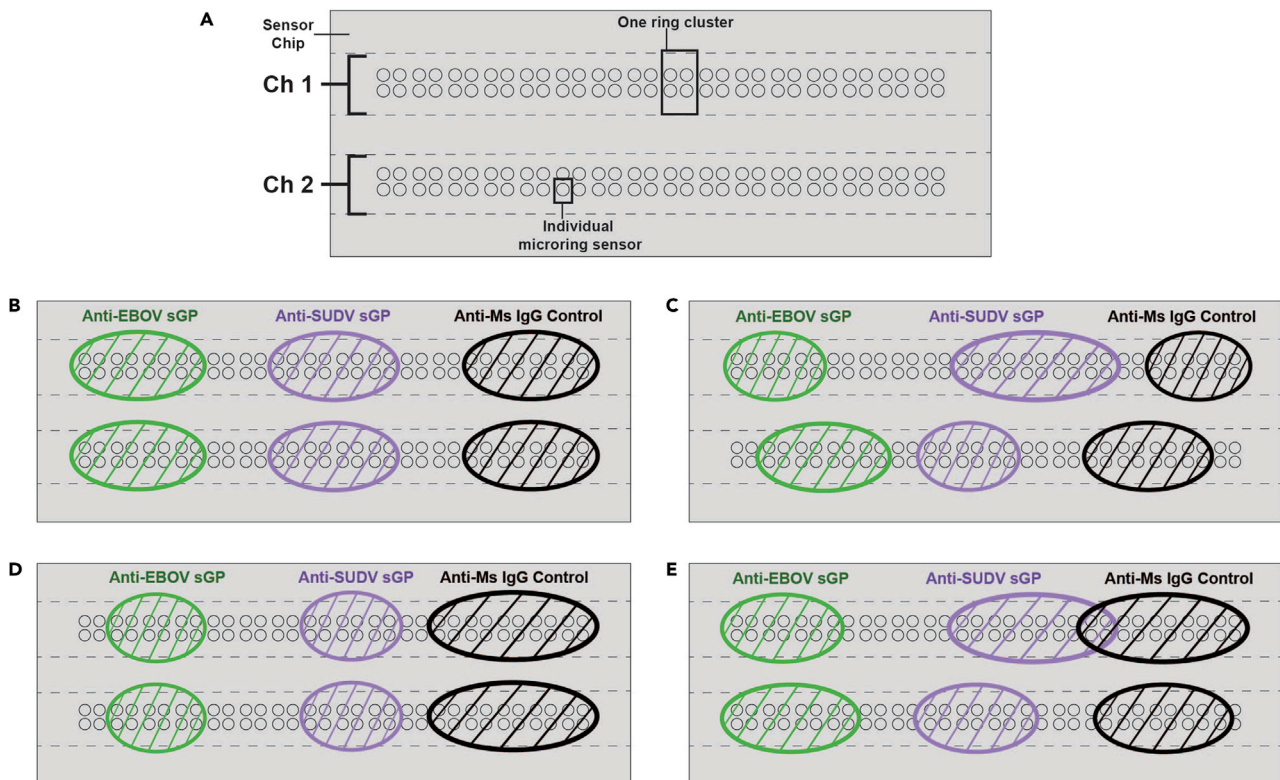
**△ CRITICAL:** Hold chips by the shorter sides to avoid damaging important chip features. Be gentle with the chips, do not drag the tweezers over the surface. Any chips or scratches can damage optical waveguides on the chip. If this occurs, discard the chip as it is no longer reliable to use.

BS3 solution should be made as close to use as possible to avoid hydrolysis. In this procedure, it is recommended that there are no stopping points once silanization begins to avoid loss of chip functionality.

**Note:** Before moving to calibration and analysis of the target in samples, it is important to validate that the antibody sandwich works on the assay platform. This includes optimizing the concentration of standard protein that elicits a saturated response and optimization of the biotinylated tracer antibody to ensure high response with minimal off-target binding. Additionally, the control rings should demonstrate minimal wavelength shift from addition of target antigen in comparison to a blank with no target present.

**▮▮▮ Pause point:** Once the dry coat has been placed on the chip and they are stored, the chips will be stable for 1–6 months depending on antibody stability.

**Optional:** This protocol has been optimized for the intended use with our specific instrumentation and experimental needs. However, there are varying options that could be explored for other applications, such as choice of silane, linker molecule, and concentration of capture



**Figure 3. Diagram of sensor array on microring chips**

(A) Blank template highlighting the 128 individual sensors in clusters of four and the two-channel arrangement.

(B–D) Examples of appropriate spotting maps. Each of the capture antibodies in the tri-plex assay encompass at least two full chip clusters and none have merged.

(E) Example of errors in chip spotting. In channel 1, the SUDV sGP antibody has merged with the control antibody. In channel 2, uneven spotting of ring clusters has occurred, with some clusters only have two of the four rings covered. This channel is still usable, as the unevenly spotted clusters can be ignored in the final data work-up, while keeping the fully spotted clusters for analysis.

reagent. Additionally, other biorecognition molecules, such as DNA, can be used in place of antibodies.

### Preparing calibration standards and samples

⌚ Timing: 30–60 min

This section sets up the reagents for the immunoassay, including how to construct calibration standards, how to prepare biological samples, and how to prepare the amplification assay reagents.

#### 4. Serial Dilutions for Calibration Standards.

- Prepare 3,600  $\mu\text{L}$  of stock pooled healthy human serum at concentration of interest by adding PBS-BSA buffer and pooled healthy serum to a labeled 5 mL Eppendorf tube.
  - 1% serum: 36  $\mu\text{L}$  serum + 3,594  $\mu\text{L}$  PBS-BSA.
  - 10% serum: 360  $\mu\text{L}$  serum + 3,240  $\mu\text{L}$  PBS-BSA.
- Invert the Eppendorf tube five times to mix.
- Prepare eight 1.5 mL microcentrifuge tubes labeled 1–8.
- In tube 2, add 300  $\mu\text{L}$  of diluted serum made in step 4a. In tubes 3–8, add 480  $\mu\text{L}$  of diluted serum.



**Table 1. Calibration standard preparation for EBOV sGP and SUDV sGP in serum matrix**

Tube #	Final concentration	Buffer dilution	Standard protein
1	2,400 ng/mL	7 $\mu$ L serum 659.4 $\mu$ L PBS-BSA OR 70 $\mu$ L serum 596.4 $\mu$ L PBS-BSA	16.8 $\mu$ L recombinant SUDV sGP (100 $\mu$ g/mL) 16.8 $\mu$ L recombinant EBOV sGP (100 $\mu$ g/mL)
2	1,200 ng/mL	300 $\mu$ L of diluted serum at corresponding dilution	300 $\mu$ L previous concentration
3	240 ng/mL	480 $\mu$ L of diluted serum at corresponding dilution	120 $\mu$ L previous concentration
4	48 ng/mL	480 $\mu$ L of diluted serum at corresponding dilution	120 $\mu$ L previous concentration
5	9.6 ng/mL	480 $\mu$ L of diluted serum at corresponding dilution	120 $\mu$ L previous concentration
6	1.92 ng/mL	480 $\mu$ L of diluted serum at corresponding dilution	120 $\mu$ L previous concentration
7	0.384 ng/mL	480 $\mu$ L of diluted serum at corresponding dilution	120 $\mu$ L previous concentration
8	Blank	330 $\mu$ L Diluted serum at corresponding dilution	0

- e. In tube 1, add 16.8  $\mu$ L each of thawed EBOV sGP standard and SUDV sGP standard (both standards stored at 100  $\mu$ g/mL in  $-80^{\circ}$ C), pooled healthy serum and PBS-BSA buffer. The amount of serum and buffer varies for each calibration, as follows, for a total of 700  $\mu$ L with each protein at 2.4  $\mu$ g/mL.
    - i. 1% serum: 7  $\mu$ L healthy serum + 659.4  $\mu$ L PBS-BSA.
    - ii. 10% serum: 70  $\mu$ L healthy serum + 596.4  $\mu$ L PBS-BSA.
  - f. Close tube 1 and invert five times to mix. Remove 300  $\mu$ L from tube 1 by pipetting up and down three times in tube 1 and then dispense by pipetting up and down three times into tube 2. Change pipette tip.
  - g. Close tube 2 and invert five times to mix. Remove 120  $\mu$ L from tube 2 by pipetting up and down three times in tube 2 and then dispense by pipetting up and down three times into tube 3. Change pipette tip.
  - h. Repeat step 4g with the next chronological tube until tube 7 is reached. Do not add protein standard into tube 8, as this will serve as the matrix blank. Steps 4c-h are summarized in [Table 1](#).
5. Sample Preparation.
- a. Thaw the frozen sample to room temperature ( $18^{\circ}$ C– $22^{\circ}$ C).
  - b. In a microcentrifuge tube, add PBS-BSA and thawed sample depending on which dilution is being prepared. In the corresponding study ([Qavi et al., 2022](#)), each sample was prepared at 10 $\times$  and 100 $\times$  in a total volume of 350  $\mu$ L. A minimum of 330  $\mu$ L is required on the Maverick Matchbox instrument.
    - i. 100 $\times$  dilution: 3.5  $\mu$ L sample + 346.5  $\mu$ L PBS-BSA buffer.
    - ii. 10 $\times$  dilution: 35  $\mu$ L sample + 315  $\mu$ L PBS-BSA buffer.
6. Prepare Detection Reagents.
- a. In a 5 mL Eppendorf tube, make a tracer antibody solution at 2  $\mu$ g/mL by adding 2889.2  $\mu$ L of PBS-BSA and 10.8  $\mu$ L of the pan-EBOV/SUDV biotinylated tracer stored at 0.5 mg/mL. This is enough tracer for two channels on four chips, which is one full 8-point calibration, or four samples analyzed at two dilutions.
  - b. Close the tube and invert five times to mix.
  - c. In a 5 mL Eppendorf tube, make a streptavidin-horse radish peroxidase (SAHRP) solution at 3  $\mu$ g/mL by adding 2891.3  $\mu$ L of PBS-BSA and 8.7  $\mu$ L of SAHRP stored at 1 mg/mL. This is enough SAHRP for two channels on four chips, which is one full 8-point calibration, or four samples analyzed at two dilutions.
  - d. Close the tube and invert five times to mix.

**Note:** Store all prepared reagents at  $4^{\circ}$ C until ready for use. Reagents (calibration standards, SAHRP, tracer) should be prepared the day of the experiment. The samples should be diluted and analyzed within 8 h of thawing. It is best to prepare smaller batches (enough for 2–4 chips at a time) to avoid any potential degradation or wasting of reagents.

**Table 2. 96-well plate set up for immunoassay on Matchbox instrument**

	1	2	3	4	5...12
A	PBS-BSA	PBS-BSA	4CN	4CN	
B	PBS-BSA	PBS-BSA	PBS-BSA	PBS-BSA	
C	Sample #1	Sample #2			
D	PBS-BSA	PBS-BSA			
E	bt-Ab	bt-Ab			
F	PBS-BSA	PBS-BSA			
G	SAHRP	SAHRP			
H	PBS-BSA	PBS-BSA			

**Optional:** The concentration of standard protein to reach the saturating condition and the concentration of the biotinylated tracer antibody were previously optimized for our experimental parameters. When optimizing new targets, a range of 300–500 ng/mL for the protein standard and 2 µg/mL for the tracer are appropriate starting points. Adjustments can be made based on initial optimization experiments.

### Running calibration standards and samples on microring instrument

⌚ **Timing:** 10 min prep work, 40 min of assay run time per sample

This section explains how to plate the reagents prepared above for loading into the Genalyte Maverick Matchbox, as well as how to run the instrument interface.

7. Preparation of Reagents in 96-Well Plate.
  - a. Collect all reagents: PBS-BSA, calibration standards or diluted samples, diluted tracer antibody, diluted SAHRP, and 4-chloro-1-naphthol (4CN).
  - b. Fill a 96-well plate according to the diagram in [Table 2](#). This plate layout corresponds to the reagent positions in the example instrument method in [Table 3](#). With this configuration, the pumps draw liquid for channel 1 in the left well (columns 1 or 3) as it draws liquid for channel 2 in the right well (columns 2 or 4). It then moves from row A to row B and so forth.
  - c. Calibrations were analyzed as follows:
    - i. Experiment #1- channel 1: calibration tube 1, channel 2: calibration tube 8.
    - ii. Experiment #2- channel 1: calibration tube 7, channel 2: calibration tube 2.
    - iii. Experiment #3- channel 1: calibration tube 3, channel 2: calibration tube 6.
    - iv. Experiment #4- channel 1: calibration tube 5, channel 2: calibration tube 4.
  - d. Samples were analyzed as follows:
    - i. Day #1 of experiments- channel 1: 10× dilution, channel 2: 100× dilution.
    - ii. Day #2 of experiments- channel 1: 100× dilution, channel 2: 10× dilution.
  - e. Return reagents to 4°C until next assay set up.
  - f. Bring filled 96 well plate to instrument and slide into well-plate holder.
8. Preparation of Microring Chip.
  - a. Remove one of the spotted chips from the storage gel pack.
  - b. Rinse chip with deionized water and dry with compressed air.
  - c. Insert the chip into the cartridge holder using the chip holder tool.
  - d. Use tweezers to push the chip into cartridge to ensure snug fit. Blow compressed air into the channels to free any remaining debris or liquid.
  - e. Put cartridge/chip into instrument with the tubes towards the front of the holder.
9. Preparation of Instrument.
  - a. Open the instrument software interface.
  - b. In 'Register & Batch Run' tab, select recipe file (see [Table 3](#) for example recipe). The recipe file needs to be in .csv format.

**Table 3. Example recipe for immunoassay on Matchbox instrument**

96-Well plate row	96-Well plate column	Flow rate (μL/min)	Duration (min)	Back forth	Comment
1	1	30	5	0	Pre-buffer rinse
Start_scan					
2	1	30	2	0	PBS-BSA
3	1	30	7	0	Analyte
4	1	30	2	0	PBS-BSA
5	1	30	7	0	Biotinylated Tracer Ab
6	1	30	2	0	PBS-BSA
7	1	30	7	0	SA-HRP
8	1	30	2	0	PBS-BSA
1	3	30	7	0	4CN
2	3	30	2	0	PBS-BSA
Stop_scan					
end_recipe					

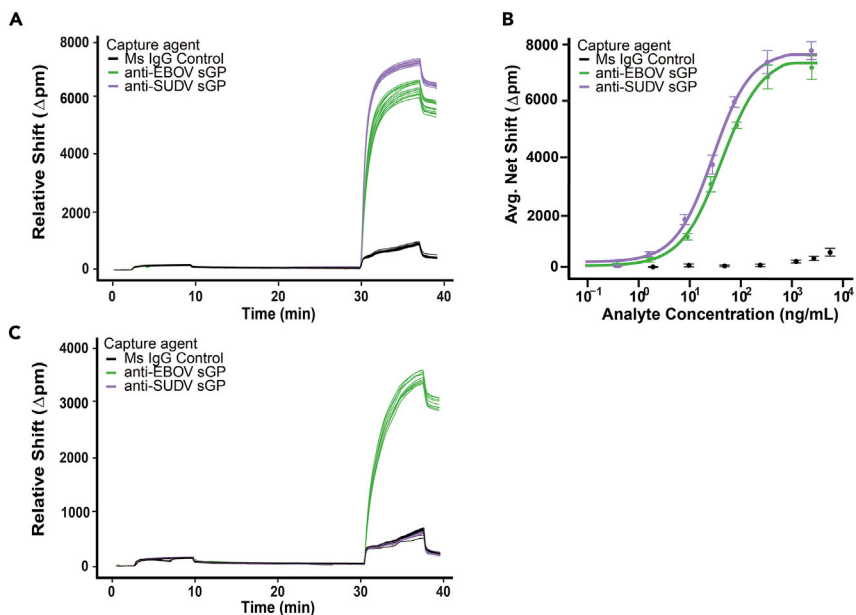
- c. Select data path (where data is to be stored after the experiment ends).
  - d. Enter chip name/identifier.
  - e. Click execute, close the door of the instrument as the chip registers.
  - f. After registration, the pumps will engage, which starts the fluid flow across the chip. Once this happens, it is helpful to reopen the instrument and observe the liquid flowing through the cartridge across both channels. Close the door before the scan starts and avoid opening it until the end of the experiment. Troubleshooting [problem 3](#).
  - g. Clean up area and prepare for next experiment.
    - i. Remove next sample from the freezer and put with the other reagents in the 4°C to thaw slowly.
    - ii. The microring chip, cartridge holder, and any other disposable materials that touched the serum should be discarded as biohazardous waste.
10. Repeat steps 7–9 for all calibration standards and samples.

## EXPECTED OUTCOMES

For an immunoassay with both standards, an example trace post work-up of data is seen in [Figure 4A](#). This is an example of tube 2 (1,200 ng/mL) for both EBOV sGP and SUDV sGP in 10% serum. Both sets of spotted rings respond to their corresponding antigen standard and the control rings have very low response. The bulk shift seen between 2 and 9 min corresponds to the analyte in the complex matrix flowing across the sensors. Once the extraneous component of the complex matrix is washed away in the buffer step, the response returns to baseline. The bulk shift between 30 and 37 min is due to the 4CN amplification reagent flowing across the surface. This solution has a different refractive index in comparison to the previous immunoassay steps, which are mainly composed of PBS-BSA. This causes a shift in resonant wavelength in all the rings. However, once the surface is rinsed with buffer, a new baseline is reached, and binding is identified in the rings spotted with the anti-EBOV sGP and anti-SUDV sGP antibodies.

An example 8-point calibration curve for EBOV sGP and SUDV sGP in a 10% serum matrix is seen in [Figure 4B](#). The antigen concentration tested should span from a saturating range to a concentration at or below the limit of detection. Additionally, it is important to have points within the curve region and close to the center of the curve, as that region is where the assay is the most sensitive.

An example immunoassay of a sample containing EBOV sGP analyzed at a 10× dilution is seen in [Figure 4C](#). While similar in profile to the standards example curve, only the rings spotted with



**Figure 4. Example data post-work up**

(A) Trace of wavelength shifts over the course of an immunoassay experiment detecting 1,200 ng/mL of EBOV sGP and SUDV sGP. Each line represents the resonant wavelength of one sensor at the specific time of experiment.

(B) Calibration curve of the EBOV sGP and SUDV sGP spanning from 0 ng/mL to 2,400 ng/mL in 10% serum. Error bars result from triplicate measurements.

(C) Trace of wavelength shift over the course of a sample containing EBOV sGP.

anti-EBOV sGP respond. The anti-SUDV sGP rings result in bulk shifts and a final shift indistinguishable from the control spotted rings.

## QUANTIFICATION AND STATISTICAL ANALYSIS

⌚ Timing: 5 min per sample, 20–30 min for final conversion to analyte concentration

This section starts with description of data output and then the data analysis for this specific project. The data was analyzed using R code modified from in-house developed code that is in a GitHub repository. The general usage of the code can be found in BaileyLabUM/biosensor on GitHub.

### 1. Data Output and Work-up for Individual Experiments.

- a. The data output from the instrument is .csv files for each individual sensor ring. They contain the resonant wavelength (nm) at a specific time point, along with the time stamp.
- b. To work up this data, the information in BaileyLabUM/biosensor GitHub repository is followed. First, create a 'groupnames\_allclusters' file (found in BaileyLabUM/MRR repository) that correlates ring numbers with capture antibody spotted on them. Name each ring using the annotated spotting map from Figure 3A (from step 3e). For partially spotted clusters or for empty rings, they can be named "Ignore" in the file names and the code skips them.
- c. For calibration data, input the concentration of target standard run in each channel in the 'Concentration' column in this file. Leave this value as '1' for unknown concentrations.
- d. Analyze each individual experiment using "analyzebiosensor" code (found in BaileyLabUM/biosensor repository).
  - i. This code first aggregates all the individual .csv files from an experiment, names each ring based on the manually annotated 'groupnames\_allclusters' file, subtracts the thermal

control sensor ring response from all rings, and then calculates the net shifts. Troubleshooting [problem 4](#).

- ii. The net shifts are calculated by subtracting the resonant wavelength prior to the amplification step from the resonant wavelength after the post-amplification buffer rinse. Using the recipe in [Table 3](#), the net shift is calculated as the difference in resonant wavelength between minute 28 and minute 41.
- iii. The average and standard deviation of net shifts across the identically named rings is then calculated. The code outputs this set of net shift data for each channel in .csv files. Additionally, the code outputs multiple plots for data visualization, such as a trace plotting the resonant wavelength over time of assay ([Figures 4A and 4C](#)) and bar graphs of the net shift for each individual ring. Troubleshooting [problem 5](#).

## 2. Calibration Data Work-up.

- a. Once each individual experiment is analyzed following step 1 and all 8 points of the calibration curve are done, complete the following to construct the calibration curve and calculate limits of detection and quantitation.
- b. Modify the 'calibrationstation' code (from BaileyLabUM/biosensor) for each specific target. Here, we specified EBOV sGP and SUDV sGP were the targets for calibration.
  - i. The code aggregates the net shifts and manually entered concentrations for all the calibration standards.
  - ii. The net shifts and concentration data for each target are fit to the following logistic function as previously described by [Robison and Bailey \(2017\)](#):  $y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2$ , where y is the

net shift ( $\Delta$  pm) of the sample of concentration  $x$  (pM),  $A_1$  is the minimum net shift ( $\Delta$  pm),  $A_2$  is the maximum net shift ( $\Delta$  pm),  $x_0$  is the center value (pM), and  $p$  is the power parameter affecting the slope around the inflection point.

- iii. After fitting the data to this function, the code outputs the fit parameters ( $A_1$ ,  $A_2$ ,  $x_0$  and  $p$ ) in a .csv file and plots the fit, along with each data point in the calibration curve on a log scale ([Figure 4B](#)).
- iv. A four parametric logistic fit is chosen for direct comparison to traditional plate-based ELISA assays. Bioassays dependent on protein-protein interaction equilibriums result in non-linear responses upon analyte/ligand binding ([Azadeh et al., 2017](#)). The lower asymptote results from concentrations below the method's limit of detection in which no net shift response is seen over a range of concentrations. The upper horizontal asymptote results from concentrations above the saturating point of the capture antibodies where any additional antigen will not affect the response and may even decrease the response due to the hook effect. The ideal working range is the linear dynamic range of the curve, with midpoint of the curve providing the area of highest sensitivity.
- c. Calculate the limit of detection (LOD) and limit of quantitation (LOQ) from the fit parameters, the average net shifts, and standard deviations from the matrix blank (tube 8 in the calibration).
  - i. For LOD calculations, add the net shift from the matrix blank to 3 times the standard deviation. Using this calculated net shift as 'y', the fit parameters, and the equation in step 2b, the concentration 'x' can be calculated. This concentration is the LOD.
  - ii. Similarly, for LOQ, take the same steps, but multiply the standard deviation of the blank by 10. Continue through to calculate the concentration 'x' based on this higher net shift 'y'. This concentration is the LOQ.
- d. Repeat this calculation for each target in each calibration.

## 3. Conversion of Net Shift to Target Concentration.

- a. Once all samples have been analyzed and worked up as described in step 1, use the 'aggdata' code in BaileyLabUM/biosensor repository to complete the following tasks. This analysis could also be done manually using basic excel functions.
- b. **Aggregation of data for each sample:** the average net shifts files created in step 1d are extracted for both channels per experiment. A new file is created that binds this aggregated

net shift data with sample information including experiment number, subjectID, and dilution ID (10× or 100×) based on the name of the data file.

- c. **Aggregation of all sample data:** this new file that was created for every experiment in 3b is then extracted and aggregated with the files from all samples.
- d. **Conversion to concentration:** the fit parameters from step 2b iii need to be manually input into the 'aggdata' code for each target in both serum matrices. The four parametric logistic fit is used to convert each net shift to concentration (ng/mL).
- e. **Selection of dilution:** the data is sorted by experiment and by target. If two dilutions are present, the net shift closest to the inflection point of the calibration curve (where the assay is most sensitive) is kept, while the other dilution is removed from the data file.
- f. **Consider dilution factor:** depending on which dilution was chosen, the concentration is multiplied by the dilution factor (10× or 100×). At this point, each target in each sample has one final concentration associated with it.
- g. **Analysis:** Further analysis can be conducted on these final concentrations depending on what is necessary for the study.

### LIMITATIONS

There are several limitations for this protocol. Assay response will vary based on the antibodies used and their respective affinities. This protocol also uses BS3 as a linker to covalently attach antibodies to the silanized sensor surface. As BS3 reacts with primary amines, this results in attachment of antibodies to the sensor surface in random orientations. This protocol also is designed for a two-step sandwich assay - primary detection of analytes in complex media may prove difficult given that the Maverick Matchbox platform is sensitive to changes in refractive index. While this assay has the potential to be used within a BSL3 or BSL4 facility, this protocol has currently only been performed in a BSL2 facility which necessitates irradiation of the samples, as well as significant precautions for user safety.

### TROUBLESHOOTING

#### Problem 1

Spill-over of capture agents while spotting.

This occurs when the drop of antibody capture agent of one antigen merges with that of a second antigen (step 3d). This can cause cross-reactivity and cannot be used reliably. This merging of spots is visualized in [Figure 3E](#), channel 1.

#### Potential solution

If this occurs, do not use that chip, as it is not reliable. Practice spotting using water/glycerol solutions before using valuable reagents to understand the mechanics and pipette tip placement. If this continues to occur, additional steps would be to decrease the volume of reagent being spotted (i.e., down to 0.2 µL) or increasing the glycerol content from 5% to 10% in the spotting solution.

#### Problem 2

Uneven spotting of ring clusters.

This occurs when the drop of antibody capture agent does not encapsulate entire ring clusters or rings themselves (step 3e). Even spotting looks like [Figures 3B–3D](#) while uneven spotting looks like [Figure 3E](#), channel 2.

#### Potential solution

In the groupnames\_allclusters file, type "Ignore" as the ring name for any clusters that are known to be partially spotted. This removes these rings from any data work up so if there is signal drop-off due to the variation in antibody coverage, it will not affect the results. Continue to use the chip, providing each capture antibody still spans at least 2–3 ring clusters.

### Problem 3

Clogging of microfluidic lines during experiment.

This occurs when a particle larger than the inner diameter of the instrument tubing gets stuck, causing a stoppage of flow of reagents across the chip and loss of data for that channel (step 9f).

### Potential solution

Avoid using samples with particulates. If samples are particularly dirty, try briefly centrifuging them and only using the resulting supernatant. If clogging occurs, be sure to purge the lines of the instrument with water before starting the next sample. This would dislodge the blockage. It is good practice to purge the microfluidic lines with bleach and water after each day of running serum samples.

### Problem 4

Malfunctioning microring sensors.

Individual or clusters of microring sensors will sporadically malfunction. This could be due to a fabrication error with the specific sensor, a small alignment error, or other reasons (step 1d in [Quantification and statistical analysis](#)).

### Potential solution

Continue letting the experiment run. During data work up, analyze all rings, then using one of the resulting csv files, identify which ring or ring cluster resulted in an abnormal trace or shift. In the `groupnames_allclusters` file, type "Ignore" for the ring(s) and reanalyze the data. This will remove the rogue ring(s) and results will not be affected.

### Problem 5

High control ring response.

If the control spotted rings are higher than they typically are (~500 pm net shift), the antibodies on the chip might have gone bad or there could have been capture reagent merging during spotting (step 1d in [Quantification and statistical analysis](#)).

### Potential solution

The data from this experiment should not be used, as the validity of the chip will be in question. If sample remains, rerun it on a different chip. If problem persists, respot and use fresher chips. If this occurs during target validation, there could be some non-specific binding of target antigens or tracer antibodies and new reagent sets should be explored.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Ryan C. Bailey ([ryanCB@umich.edu](mailto:ryanCB@umich.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate datasets.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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## AUTHOR CONTRIBUTIONS

K.M., A.J.Q., M.J.A., F.W.H., D.W.L., L.Y., G.K.A., and R.C.B. conceived this study. NHP studies were performed by M.J.A., H.V., L.Z., J.D., J.F., and F.W.H. Microring studies were performed by A.J.Q. and K.M. Data analysis was performed by A.J.Q., K.M., R.C.B., and G.K.A. Initial manuscript draft was written by K.M. and A.J.Q. and edited by G.K.A. and D.W.L. with input from all authors.

## DECLARATION OF INTERESTS

R.C.B. has minor financial interest in Genalyte, Inc. M.J.A., H.V., and F.W.H. are employees of Integrated BioTherapeutics, Inc. L.Z. is co-owner of Mapp Biopharmaceutical, Inc.

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