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Evaluation of the Octet Biolayer Interferometer Platform for Biological Agent Environmental Sampling

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14. ABSTRACT:
The U.S. Army Edgewood Chemical Biological Center (now the U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) examined the Octet RED384 and HTX instruments (Pall ForteBio; Fremont, CA) to determine the feasibility of using high-throughput, label-free, real-time measurements for analysis of biological molecules by way of biolayer interferometry (BLI). The commercial off-the-shelf Octet platforms were challenged with six bioagents spiked into two environmental sampling matrices, namely, phosphate-buffered saline with 0.1% Triton X-100, and dry filter unit extract. *Ricinus communis* agglutinin, T2 mycotoxin, *Bacillus anthracis*, NNR-1 spores, *Francisella tularensis* var. *tularensis*, *Yersinia pestis* EV76, and Ebola virus-like particles were selected to determine protein–protein interactions for small (mycotoxin) and large (virus and spore) particles. BLI technology examined limits of detection and true-positive and false-negative responses for the bioagents. Octet biosensors were configured in 96- and 384-well microplates using monoclonal antibodies immobilized to commercially available Dip and Read coupling kits (Pall ForteBio). Antibodies were functionalized to three Octet biosensors that had surface chemistries designed to analyze specific biomolecular interactions: streptavidin, amine reactive second generation, and anti-mouse Fc capture. Overall, the Octet system enabled high-throughput sample analysis with relatively few manipulations and provided a comprehensive data output.

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PREFACE

The work described in this report was started in June 2017 and completed in June 2018.

At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CDC) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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EVALUATION OF THE OCTET BIOLAYER INTERFEROMETER PLATFORM FOR BIOLOGICAL AGENT ENVIRONMENTAL SAMPLING

1. INTRODUCTION

A team from the U.S. Army Edgewood Chemical Biological Center (now known as the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC; Aberdeen Proving Ground, MD) evaluated the Octet RED384 and HTX instruments (Pall ForteBio; Fremont, CA) in two environmental sampling matrices spiked with six biological agents: *Ricinus communis* agglutinin (RCA 120), T2 mycotoxin, *Bacillus anthracis* NNR-1 spores, *Francisella tularensis* var. *tularensis*, *Yersinia pestis* EV76, and Ebola virus-like particles (VLPs). The objective of the evaluation was to examine the commercial off-the-shelf Octet platforms using high-throughput, label-free, real-time measurements for analysis of biological molecules by way of biolayer interferometry (BLI). The Octet biosensors were configured in 96- and 384-well microplates and were not subject to sample-throughput limitations caused by system microfluidics. For this test evaluation, the applications included determination of protein–protein interactions for small (mycotoxin) and large (virus and spore) particles using BLI technology to examine limits of detection (LODs) and true-positive and false-negative responses for the biological agents. In addition, monoclonal antibodies from the Defense Biological Product Assurance Office (DBPAO; Aberdeen Proving Ground, MD) were immobilized using commercially available Pall ForteBio Dip and Read coupling kits to functionalize Octet biosensors that had surface chemistries designed to analyze specific biomolecular interactions. The three biosensor chemistries selected for this evaluation were streptavidin (SA), amine reactive second generation (AR2G), and anti-mouse Fc capture (AMC). The results from this Octet platform evaluation will be used to provide insight into the feasibility and merit of structuring potential follow-on investigations to further optimize the Octet performance data for biological agents in environmental sampling testing scenarios.

1.1 Scope

The Octet platform was selected to examine the feasibility for conducting high-throughput analysis of biomolecular interactions in a 96- and 384-well plate format for environmental sampling scenarios. Of particular interest was the ability to support in-house method development by purchasing commercially available reagent coupling kits from a manufacturer as opposed to relying on contractual outsourcing. The analysis was focused on immobilizing DBPAO proteins of interest to the various biosensor surfaces using simplified coupling chemistries from the Dip and Read kit applications. Having an in-house capability to produce customized, high-throughput biosensor microplates at a significant cost reduction was important during this evaluation. Toward this end, efforts were made to gauge the "methods and materials" processes, to help factor in a rough order of magnitude (ROM) estimate of the relative cost per assay for reagents and to ascertain the potential for method-friendly manipulations for in-house sensor development. In addition, it was necessary to identify the skill level required for laboratory personnel to perform in-house sensor manufacturing. This capability allows for mission-specific "designer" microplate sensors to be customized in-house, thereby precluding the delays and contractual interruptions that often occur when materials are outsourced.

1.2 Description of Pall ForteBio Octet System

The Octet platform provides real-time, label-free analysis coupled with multiplexed biosensor formats that monitor the binding of protein interactions in real time while performing sample analysis and processing for up to 96 or 384 samples in parallel. Protein quantitation for 96 samples can generally be completed in as little as 2 min with assay throughput enabled by multiplex biosensor formats. Two environmental media were used in this analysis: phosphate-buffered saline with 0.1% Triton X-100 (PBS-T) and a dry filter unit (DFU) sample matrix that was extracted from filters that had been used to collect particulates from ambient air. No sample preparation was performed during this evaluation because the Octet system was purported to have the ability to test proteins in crude mixtures, which drastically reduces the need for sample preparation. The Octet instruments used in this evaluation were the RED384 system for analyzing antibody–antigen binding and the HTX system for loading antibodies onto the sensor and soaking the sensors.[1]

Figure 1. (Left) Octet RED384 16-channel instrument provides simultaneous sampling; (right) Octet HTX instrument simultaneously monitors up to 96 biosensors.

The Octet system incorporates BLI label-free technology for measuring biomolecular interactions. The system uses an optical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein (on the biosensor tip) and an internal reference layer. The interference pattern shifts when there is a change in the number of molecules bound to the biosensor tip, which is measured in real time.

For this effort, the binding between selected DBPAO monoclonal antibodies (immobilized on the biosensor tip surface) with their respective analytes (in solution) produced an increase in optical thickness at the biosensor tip that resulted in a distinguishable wavelength shift. The change in thickness enabled direct measurement of the biological layer when the binding or dissociation of the biological agent from the biosensor caused a shift in the interference pattern. The shift generated a response profile on the Octet system as the interactions were measured in real time (Figure 2).[2]

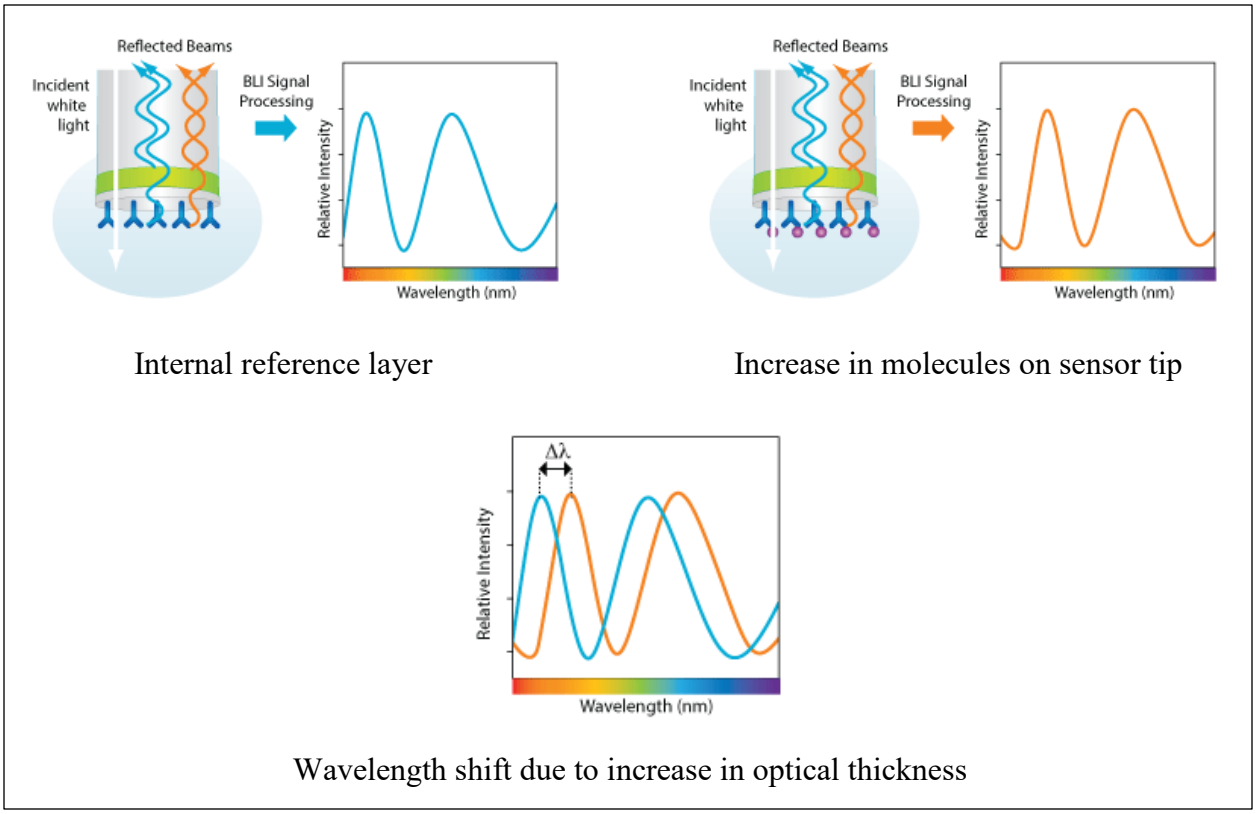

Figure 2. Measurement of biomolecular interactions using label-free Octet biolayer interferometry technology. Δλ, wavelength shift.

1.3 Dip and Read Biosensors for Assay Development

The biomolecular interactions for DBPAO-selected monoclonal capture antibodies were evaluated with their respective antigens using the RED384 and HTX systems (Table 1). The testing on the Octet system was designed to include monoclonal antibodies that are currently being used to develop gold-standard biological agent detection assays for various sampling scenarios. For the Octet system evaluation, antibodies were immobilized in-house onto Dip and Read biosensors configured into 96-well plates. Assay kits for three biosensors that had SA, AMC, and AR2G surface chemistries were used to functionalize the proteins for specific biomolecular interactions (Table 2).

Table 1. Monoclonal Antibody and Antigens Used in the Octet Evaluation

Antibody	DBPAO Product Catalog Number	Antigen	Source	Lot Number
Anti-ricin	AB-RIC-MAB1	*R. communis* (RCA 120)	Sigma-Aldrich (St. Louis, MO)	L-7886 (5 mg/mL)
Anti-T2	AB-T2T-MAB1	T2 mycotoxin		T4887 (25 mg)
Anti-Ebola	AB-EB-MAB3	Ebola VLPs	IBT Bioservices (Rockville, MD)	0550-001 (1 mg/mL)
Anti-*Y. pestis*	AB-YERS-MAB1	*Y. pestis* EV76	CCDC CBC	NA
Anti-*F. tularensis*	AB-FTUL-MAB2	*F. tularensis* var. *tularensis*		
Anti-*B. anthracis*	AB-BA-MAB2	*B. anthracis* (NNR-1 spores)		

NA, not applicable.

Table 2. Octet Dip and Read Biosensors

Dip and Read Biosensor	Description
AMC	AMC enables kinetic characterization of macromolecular interactions between mouse Fc-containing proteins and target analytes. Immobilization of mouse Fc-containing proteins is achieved through a factory-immobilized anti-mouse Fc-specific antibody with high affinity for the mouse Fc domain that provides the stable baseline required for demanding kinetics applications. AMC biosensors can be regenerated up to 10 times via a standard low-pH protocol in as little as 2 min for select applications.
AR2G	Second-generation carboxylate functionalized surface allows covalent coupling of proteins via EDC and sulfo-NHS-mediated amide bond formation. The second-generation surface provides increased loading density, more robust loading conditions, and decreased nonspecific binding.
SA	Biological molecules are biotinylated using established protocols and immobilized onto the SA biosensor surface for custom assay development. The high sensitivity and stable baseline of the SA biosensor supports kinetic as well as quantitation experiments.

EDC, 1-ethyl-3-(3-dimethylamino) propyl carbodiimide hydrochloride; sulfo-NHS, sulfo-*N*-hydroxysuccinimide.

The following three tasks were performed under this research and development effort to evaluate Octet BLI performance using the Dip and Read biosensors: (1) establish preliminary assay development and LOD cutoffs for six biological assays of interest, (2) downselect the Octet sensor demonstrating the most consistent results based on LOD and assay reproducibility (using percent coefficient of variation [%CV]), and (3) determine true-positive rate (TPR) sensitivity responses for cocktailed antigen combinations on the sensors. Throughout the evaluation, environmental milieus consisted of PBS-T- and DFU-extracted environmental matrices.

2. MATERIALS AND METHODS

2.1 Biosensor Antibody Coupling

The AMC sensor (Pall ForteBio; catalog no. 18-384, lot no. 1707052) coupling used the factory-made, immobilized anti-mouse Fc-specific antibody, which has high affinity for the mouse Fc domain. All of the DBPAO antibodies selected for this evaluation contained the mouse Fc domain, and all were all directly loaded onto the AMC sensors via a dip and load function. The antibody was coupled to the sensor tip by dipping the sensor tip into an antibody solution (prepared at a concentration of 10 µg/mL in PBS-T) for 300 s. The sensors were then quenched with NeutrAvidin biotin-binding protein (1 mg/mL; Thermo Fisher Scientific; Waltham, MA) for 300 s to eliminate any open binding sites and to ultimately decrease nonspecific binding. For testing, the antibodies were diluted to a concentration of 25 µg/mL in a PBS-T- or DFU-extracted environmental matrix.

The AR2G sensor (Pall ForteBio; catalog no. 18-5093, lot no. 1506302) carboxylate-functionalized surface allowed covalent coupling of the DBPAO monoclonal antibodies via EDC/sulfo-NHS-mediated amide bond formation. All antibodies used for the AR2G sensor coupling were first dialyzed into PBS-T using a standard dialysis cassette with three buffer exchanges to ensure removal of amine-containing contaminants (such as Tris, ammonium sulfate, etc.). The antibodies were coupled to the sensor tip by dipping the sensor into a 20 mM EDC/10 mM sulfo-NHS activation buffer solution for 300 s. The sensors were then dipped into the antibody loading solution (25 µg/mL) for 600 s. The sensors were quenched with 1 M ethanolamine at pH 8.5 for 300 s, to eliminate any open binding sites and to ultimately decrease nonspecific binding. Because the AR2G sensors require an amine coupling reaction, the pH of buffer used to dilute the DBPAO monoclonal antibodies for loading had to be at least 1 to 1.5 points higher than that of the buffer used for immobilizing. The DBPAO monoclonal antibodies loaded optimally in an immobilization buffer of 10 mM sodium acetate at pH 5. For this study, the EDC/sulfo-NHS concentration remained constant, and all antibodies were loaded with 10 mM sodium acetate at pH 5.

For the SA sensor (Pall ForteBio; catalog no. 18-5020, lot no. 1707071) development, the antibody was coupled to the sensor tip by dipping the sensor into an antibody solution prepared at 10 µg/mL in PBS-T for 300 s. The sensors were then quenched with NeutrAvidin (1 mg/mL) for 300 s to eliminate any open binding sites and to ultimately decrease nonspecific binding. Subsequently, the DBPAO antibodies were biotinylated using EZ-Link sulfo-NHS-LC-biotin (Thermo Fisher Scientific; catalog no. 21335) and immobilized onto the Octet SA biosensor surface at 10 µg/mL for testing.

The coupling procedures for each biosensor type were developed in-house, using standard manufacturers' protocols for the antibody load concentrations and incubation times, the immobilization buffers, and the quench buffers for the DBPAO monoclonal antibodies. The coupling specifications are summarized in Table 3.

Table 3. Summary: Reagents Used for Coupling DBPAO Monoclonal Antibodies to Octet Sensor Types

Reagent	AMC	AR2G	SA
Antibody load concentration (µg/mL)	25	25	10
Sensor activation time (s)	NA	300	NA
Sensor activation buffer	NA	EDC/sulfo-NHS	NA
Sensor activation buffer concentration	NA	20 mM EDC/ 10 mM NHS	NA
Load/incubation time (s)	600	600	600
Immobilization buffer	PBS-T	10 mM sodium acetate, pH 5	PBS-T
Quench buffer	Neutravidin	Neutravidin	Neutravidin

NA, not applicable.

2.2 Microbial Pathogens and Culture Conditions

B. anthracis NNR-1 spores were prepared in PBS-T at approximately 2.00E+09 colony-forming units (cfu) per milliliter and stored at 4 °C until use. The preparation had greater than 95% spore population, and spore hardiness was confirmed by heat-shock treatment. The test samples and controls were cultured in triplicate on tryptic soy agar plates and incubated overnight at 37 °C. The bacterial colonies were counted manually. Replicate dilutions generating 20–200 colonies were computed and averaged to derive the mean log colony-forming units. Dilutions that exhibited colonies "too numerous to count" were excluded. Dilutions with less than 20 colonies were considered "too few to count".

F. tularensis was thawed from frozen stock material. One loopful of the stock was streaked for isolation onto each of two chocolate agar plates and incubated for 48 h at 37 °C. The streaked plates were checked for purity (by Gram stain) and colony morphology. Liquid cultures were prepared in Mueller–Hinton broth supplemented with IsoVitaleX enrichment (Becton, Dickinson and Company; Franklin Lakes, NJ). A stock was prepared at a concentration of 4.50E+08 cfu/mL in PBS-T and stored at 4 °C until use. Bacterial colonies were counted using the same process as mentioned for *B. anthracis* NNR-1 spores.

Y. pestis EV76 was thawed from frozen stock material. One loopful of the stock was streaked for isolation onto each of two brain–heart infusion agar plates, which were incubated for 48 h at 28 °C. The streaked plates were checked for purity (by Gram stain) and colony morphology. Liquid cultures were prepared in brain–heart infusion broth and incubated for 24 h at 37 °C to guarantee F1 protein expression. A stock was prepared at a concentration of 2.50E+08 cfu/mL in PBS-T and stored at 4 °C until use. Bacterial colonies were counted using the same process as mentioned for the *B. anthracis* NNR-1 spores.

Ebola VLPs were purchased at a concentration of 1 mg/mL from IBT BioServices (catalog no. 0550-001IBT). The RCA 120 complex at a concentration of 5 mg/mL was purchased from Sigma-Aldrich (catalog no. L7886-5 mg).

2.3 Preparation of DFU Sample Matrix

Polyester felt, dry filter disks with a 1 µm rating (Army issue, product no. 5-15-31505) were removed from designated facility air-handling systems and extracted with PBS-T to provide an environmental matrix for subsequent analysis. Each DFU was placed into a 50 mL conical tube that contained 15 mL of PBS-T. It was then vortexed for 5 min at 100 rpm (Vortex-Genie; Scientific Industries; Bohemia, NY) to release the bioparticulates. After the vortex step, the liquid contents of the conical tube were poured into a fresh tube and used throughout the evaluation as the DFU extraction matrix for spiking antigens. In some cases, the extracted matrix was slightly turbid and light yellow.

2.4 Task 1: Determine LODs

Quantitation of the assay cutoff for each bioagent was determined by generating a standard curve for the three selected biosensor types using both the PBS-T and the DFU environmental matrix buffers. Each bioagent was run in a twofold titration series of varying concentrations, and each concentration had three replicates (Table 4). All LODs were determined using the response versus sensor (index) graph as well as the affinity sensogram from each run.

Table 4. Test Construct for Octet Limit of Detection Evaluation

Bioagent	Biosensor Types	Test Matrixes	No. of Serial Dilutions	Test Replicates	Total No. of Tests
RCA 120	3	2	20	3	360
B. anthracis	3	2	8	3	144
F. tularensis	3	2	10	3	180
Y. pestis	3	2	16	3	288
Ebola VLPs	3	2	16	3	288
T2 mycotoxin	3	2	8	3	144
Total					1404

2.5 Task 2: Downselect Biosensor for Sensitivity Testing

Based on the results generated from the LOD determination, the Octet biosensor demonstrating the most consistent results for LOD and %CV was downselected for further sensitivity testing.

2.6 Task 3: Determine TPR Sensitivity Responses

Using the downselected biosensor, TPR evaluations were conducted with antigen cocktail mixtures in PBS-T and DFU environmental matrices (Table 5). Antigen concentrations were spiked into the cocktail at the assay cutoff levels identified during the LOD determinations. Each cocktail combination was run in triplicate with a parallel reference sensor for subtracting the background, a negative control (plain buffer well), and a positive-control antigen at the LOD concentration specific to each bioagent assay. The respective antibodies for each antigen in the

cocktail were loaded onto the sensors using the same standard method that was applied during the LOD determination. Each bioagent was tested in three different cocktail combinations, for a total of nine sensitivity tests per bioagent in each matrix. The TPR was calculated based on a true-positive versus a false-negative determination, whereby a test result incorrectly indicates that a particular attribute was missing. In this case, that would be the percentage of failures of the Octet sensor to detect a bioagent when the sample had been spiked with the agent.

Table 5. Test Construct for Octet Sensitivity Evaluation

Cocktail Number	Bioagents	Buffer Matrix	DFU Matrix	Number of Tests per Bioagent					
				R	T2	BA	YP	FT	EB
1	R, T2, BA, EB	1	1	3	3	3			3
2	T2, BA, FT	1	1		3	3		3	
3	BA, FT, YP, R	1	1	3		3	3	3	
4	FT, YP, EB	1	1				3	3	3
5	YP, EB, R, T2	1	1	3	3		3		3
Total				9	9	9	9	9	9

R, RCA 120; T2, T2 mycotoxin; BA, *B. anthracis*; EB, Ebola VLPs; YP, *Y. pestis*; FT, *F. tularensis*.

3. RESULTS

Statistical analyses of the raw data were computed using a geometric mean derived from *x* (the sum of values in the set) divided by the number of values, *n,* to provide equal weight to all experimentally derived values and reduce the influence of outliers. The standard deviation (SD) was calculated from the geometric mean. The %CV was calculated to represent the ratio of the SD to the mean:

$$\text{Mean} = \bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

$$\text{SD} = s_{\bar{x}} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

$$\%\text{CV} = \left|\frac{s_{\bar{x}}}{\bar{x}}\right| \times 100\%$$

where *i* represents the sample data set, *n* is the number of sample values, *x* is each sample value, and ∑ is the sum of the sample values.

3.1 Task 1: LOD Determinations for Octet Assays in PBS-T and DFU Environmental Matrices

For LOD determination, each biological agent was spiked into both PBS-T and DFU environmental buffer matrices using a twofold titration scheme with concentration ranges as shown in Table 6. Quantitation of the assay cutoff for each bioagent was determined by generating a standard curve for the three selected biosensor types using the PBS-T and DFU environmental matrix buffers. Each bioagent was run in a titration series, and each concentration had three replicates (Table 4). Each LOD was determined using the response versus sensor (index) graph as well as the affinity sensogram from each run.

Table 6. Bioagent Titration Ranges Used in PBS-T and DFU Environmental Matrices for LOD Determinations

Bioagent	Titration Range	
	PBS-T	DFU
Y. pestis	2.5E+08 – 1.5E+04 cfu/mL	2.5E+08 – 1.5E+04 cfu/mL
F. tularensis	4.5E+08 – 9.25E+05 cfu/mL	4.5E+08 – 9.25E+05 cfu/mL
B. anthracis	2.00E+09 – 1.56E+07 cfu/mL	2.00E+09 – 1.56E+07 cfu/mL
Ebola VLPs	1 mg/mL – 61 ng/mL	1 mg/mL – 61 ng/mL
T2 mycotoxin	1 mg/mL – 7.80 µg/mL	500–15.63 µg/mL
RCA 120	1 mg/mL – 238 pg/mL	1 mg/mL – 238 pg/mL

Table 7 summarizes the LOD values obtained for each biological agent spiked in PBS-T buffer matrices on the three Octet sensor types. The PBS-T preliminary data suggest that the AR2G sensor yielded a consistent LOD of 3.81 ng/mL (0% CV) for the RCA 120 assay. The SA and AMC sensors produced lower LODs (1.27 and 3.18 ng/mL, respectively); however, the trade-off was higher CVs (43 and 35%, respectively). As shown by the T2 mycotoxin LODs, the AMC sensor outperformed both the AR2G and SA sensors by yielding an LOD of 31.25 µg/mL (0% CV). For the *B. anthracis* assay, the LODs among the three sensors were comparable within two titration sequences (3.13E+07 and 6.25E+07 cfu/mL); however, the AR2G sensor provided consistent values for all three replicates at an LOD of 6.25E+07 cfu/mL (0% CV). Both the SA and the AMC sensors achieved an LOD of 3.13E+07 cfu/mL for two of the three replicates, but the CVs were high (43 and 35%, respectively). The most consistent values for the *F. tularensis* assay were seen on the AMC sensor, which demonstrated an LOD of 1.50E+07 cfu/mL (0% CV). The SA and AR2G sensors exhibited one lower titration at 7.40E+06 cfu/mL; however, the CV values were high (44 and 66%, respectively). An LOD cutoff at 7.80E+06 cfu/mL (0% CV) was consistently achieved for the *Y. pestis* assay on both the AMC and the AR2G sensors. The Ebola VLP assay produced a consistent detection at 31.25 µg/mL (0% CV) on the AR2G sensor for all three replicates. The SA and AMC sensors responded accordingly; however, the CVs were elevated due to one nonconforming replicate in each set (35 and 58%, respectively).

Table 7. Octet Sensor LOD Data Summary for Biological Agents Spiked in PBS-T Buffer

Organism in PBS-T	Replicate	Sensor Type		
		AMC	AR2G	SA
RCA 120 (ng/mL)	1	3.81	3.81	1.91
	2	1.91	3.81	0.95
	3	3.81	3.81	0.95
Mean		**3.18**	**3.81**	**1.27**
SD		1.10	0	0.55
%CV		34.50	0	43.44
T2 mycotoxin (µg/mL)	1	31.25	500.00	62.50
	2	31.25	250.00	125.00
	3	31.25	500.00	62.50
Mean		**31.25**	**416.67**	**83.33**
SD		0	144.34	36.08
%CV		0	34.64	43.30
B. anthracis (cfu/mL)	1	1.56E+07	6.25E+07	3.13E+07
	2	3.13E+07	6.25E+07	3.13E+07
	3	3.13E+07	6.25E+07	6.25E+07
Mean		**26,066,667**	**62,500,000**	**41,700,000**
SD		9,064,399	0	18,013,328
%CV		34.77	0	43.20
F. tularensis (cfu/mL)	1	1.50E+07	3.00E+07	1.50E+07
	2	1.50E+07	1.50E+07	7.40E+06
	3	1.50E+07	7.40E+06	7.40E+06
Mean		**15,000,000**	**17,466,667**	**9,933,333**
SD		0	11,500,145	4,387,862
%CV		0	65.84	44.17
Y. pestis (cfu/mL)	1	7.80E+06	7.80E+06	1.56E+07
	2	7.80E+06	7.80E+06	1.56E+07
	3	7.80E+06	7.80E+06	1.56E+07
Mean		**7,800,000**	**7,800,000**	**15,600,000**
SD		0	0	0
%CV		0	0	0
Ebola VLPs (µg/mL)	1	31.25	31.25	15.63
	2	7.81	31.25	31.25
	3	31.25	31.25	31.25
Mean		**23.45**	**31.25**	**26.04**
SD		13.53	0	9.02
%CV		57.74	0	34.63

Note: Mean values are shown in bold.

All CV values were averaged for each sensor per biological assay to determine the sensor that achieved the lowest variation in the test results in general. The AR2G sensor had the overall lowest CV (13.17%) when compared to the CVs for the AMC (25.41%) and the SA (33.09%) sensors. This value represents a total of 702 test data points that were examined for the PBS-T buffer studies for six bioagents (Table 8).

Table 8. Consolidated Average CVs for PBS-T LOD Study

Average %CV		
AMC	**AR2G**	**SA**
25.41	13.17	33.09

Table 9 summarizes the LOD values obtained for each biological agent spiked in the DFU-extracted matrices on the Octet sensor types. The DFU matrix preliminary data suggest that the AMC and SA sensors yielded similar LODs at 2.54 and 1.27 ng/mL, respectfully; however, the CVs were high (e.g., 43% for the RCA 120 assay for all three sensor types). The AR2G sensor did not perform well; the RCA 120 spiked into DFU extractions yielded an LOD of 4.45 ng/mL and a high CV of 65%. The T2 mycotoxin assay was identical for all three sensors, yielding an LOD of 500 µg/mL and a 0% CV for the data set. For the *B. anthracis* assay, the LOD for the AR2G sensor provided consistent values for all three replicates: the LOD was 3.13E+07 cfu/mL (0% CV). The AMC sensor achieved a somewhat higher LOD cutoff at 5.0E+08 cfu/mL (0% CV), and the same was observed for the SA sensor LOD at 2.50E+08 cfu/mL (0% CV). The replicate data on all three sensor types was consistent with CVs at 0%; however, the lowest LOD cutoff was seen on the SA sensor for the *F. tularensis* assay at 1.20E+08 cfu/mL (0% CV). An LOD cutoff at 1.56E+07 cfu/mL (0% CV) was achieved for the *Y. pestis* assay on the AMC sensor. The Ebola VLP assay produced a cutoff at 13.02 µg/mL (35% CV) on the SA sensor. The AMC and AR2G sensor LODs were approximately 1 to 2 titrations above that of the SA sensor LOD, and those sensors also had elevated CVs (85 and 35%, respectively).

Table 9. Octet Sensor LOD Data Summary for Biological Agents Spiked in DFU Matrix

Organism in DFU	Replicate	Sensor Type		
		AMC	AR2G	SA
RCA 120 (ng/mL)	1	1.91	7.63	0.95
	2	3.81	3.81	0.95
	3	1.91	1.91	1.91
Mean		**2.54**	**4.45**	**1.27**
SD		1.10	2.91	0.55
%CV		43.13	65.45	43.44
T2 mycotoxin (µg/mL)	1	500	500	500
	2	500	500	500
	3	500	500	500
Mean		**500**	**500**	**500**
SD		0	0	0
%CV		0	0	0
B. anthracis (cfu/mL)	1	5.00E+08	3.13E+07	2.50E+08
	2	5.00E+08	3.13E+07	2.50E+08
	3	5.00E+08	3.13E+07	2.50E+08
Mean		**5.00E+08**	**31,250,000**	**2.50E+08**
SD		0	0	0
%CV		0	0	0
F. tularensis (cfu/mL)	1	2.38E+08	7.75E+08	1.20E+08
	2	2.38E+08	7.75E+08	1.20E+08
	3	2.38E+08	7.75E+08	1.20E+08
Mean		**2.38E+08**	**7.75E+08**	**1.20E+08**
SD		0	0	0
%CV		0	0	0
Y. pestis (cfu/mL)	1	1.56E+07	7.80E+06	1.25E+08
	2	1.56E+07	3.13E+07	1.25E+08
	3	1.56E+07	6.25E+07	1.25E+08
Mean		**15,600,000**	**33,866,667**	**1.25E+08**
SD		0	27,440,177	0
%CV		0	81.02	0
Ebola VLPs (µg/mL)	1	7.81	62.50	7.81
	2	31.25	62.50	15.63
	3	7.81	31.25	15.63
Mean		**19.53**	**52.08**	**13.02**
SD		16.54	18.04	4.51
%CV		84.87	34.64	34.67

Note: Mean values are shown in bold.

All %CV values were averaged for each sensor per biological assay to identify the sensor that achieved the lowest variation in the test results. The SA sensor had the overall lowest CV (15.62%) when compared with the AMC (25.60% CV) and the AR2G (36.23% CV) sensors. This value represents a total of 702 test data points that were examined for the DFU extraction studies for six bioagents (Table 10).

Table 10. Consolidated Average CVs for DFU Matrix LOD Study

Average %CV		
AMC	**AR2G**	**SA**
25.60	36.23	15.62

Appendix A provides the Octet sensograms, which depict the wavelength shifts due to the biomolecular interactions that occurred for each bioagent in the presence of the respective antigens spiked in PBS-T- and DFU-extracted matrices. Each bioagent assay was performed in triplicate on each of three Octet sensors at the full titration series. In addition to the associated sensograms, Appendix A includes a specified range for each organism titration series, and if applicable, the kinetic analysis (the equilibrium dissociation constant, K_D, which describes the strength of the binding affinity in the antibody–antigen biomolecular interaction).

3.2 Task 2: Sensor Downselection

The results generated in Task 1 were compiled and statistically weighted to support identification of the best overall sensor to be carried forward for specificity testing in Task 3. Figures 3–5 describe the relationship between the LODs and the SDs exhibited for the PBS-T and DFU matrices. For the sensor downselection, the results for bioagents spiked in the DFU environmental matrix were given more weight than those in the PBS-T because the DFU matrix was more representative of an environmental sampling process. All three sensors performed identically for the T2 mycotoxin assay: LOD cutoffs were 500 µg/mL and CVs were 0%. The AR2G sensor outperformed the AMC and SA sensors and exhibited a *B. anthracis* LOD of 3.13E+07 cfu/mL (0% CV), as compared with 5.00E+08 and 2.50E+08 cfu/mL for AMC and SA, respectively. The SA sensor performed well for the small-molecule RCA 120 and T2 mycotoxin assays. In the end, the AR2G sensor was downselected for the Task 3 sensitivity testing. This decision was made owing to the consistent results that were obtained for the large-molecule assays, particularly *B. anthracis* NNR-1 spores, combined with the fact that the antibody coupling procedure for the AR2G sensor required only one step, as opposed to the two-step process required for the SA sensor preparation.

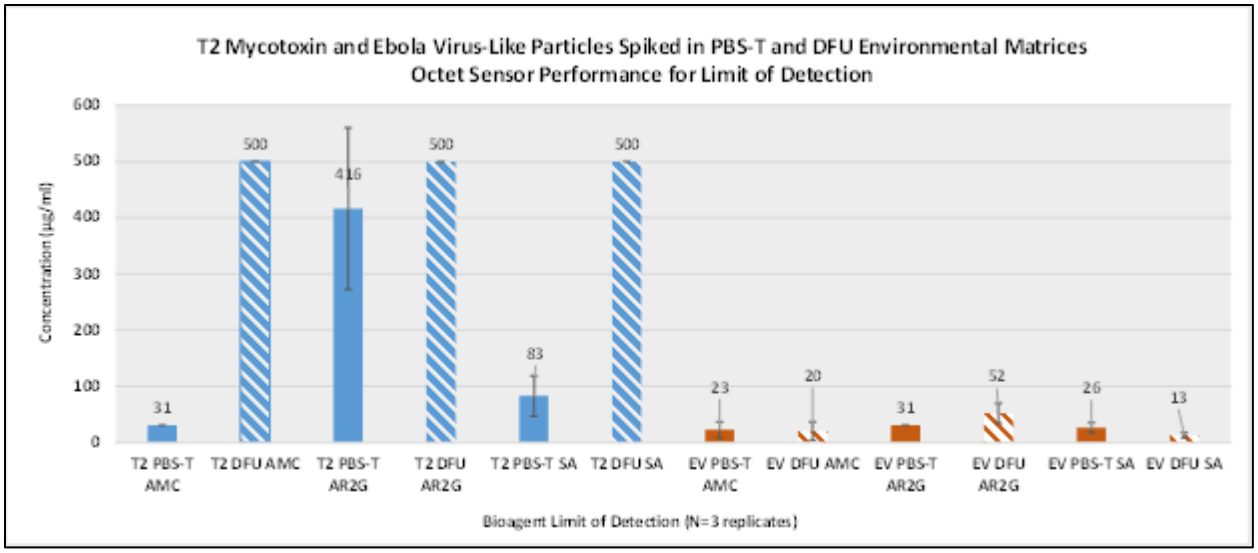

Figure 3. Octet sensor performance data for T2 mycotoxin and Ebola VLPs spiked into PBS-T and DFU environmental matrices.

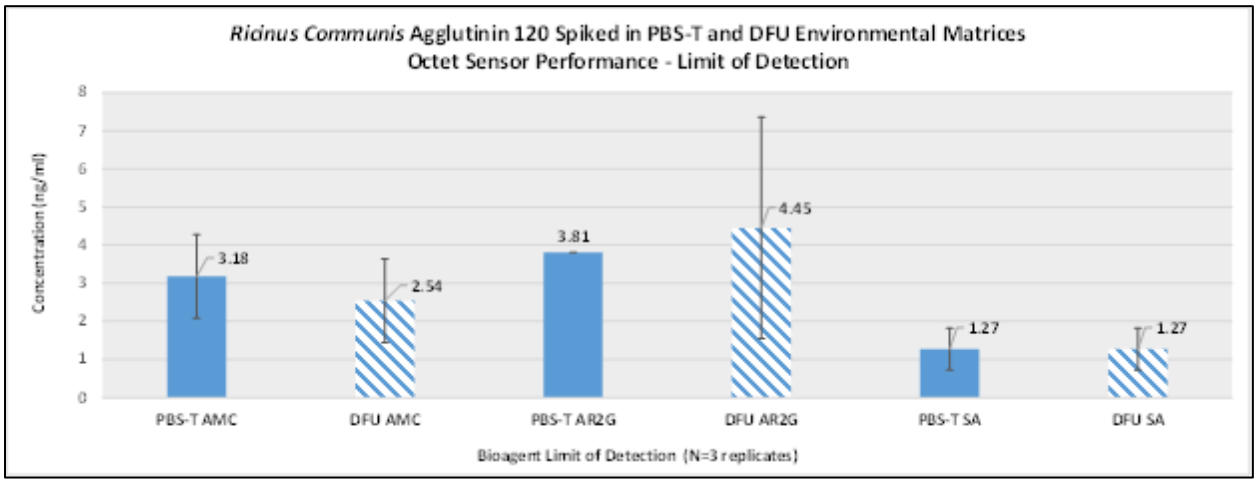

Figure 4. Octet sensor performance data for RCA 120 complex spiked into PBS-T and DFU environmental matrices.

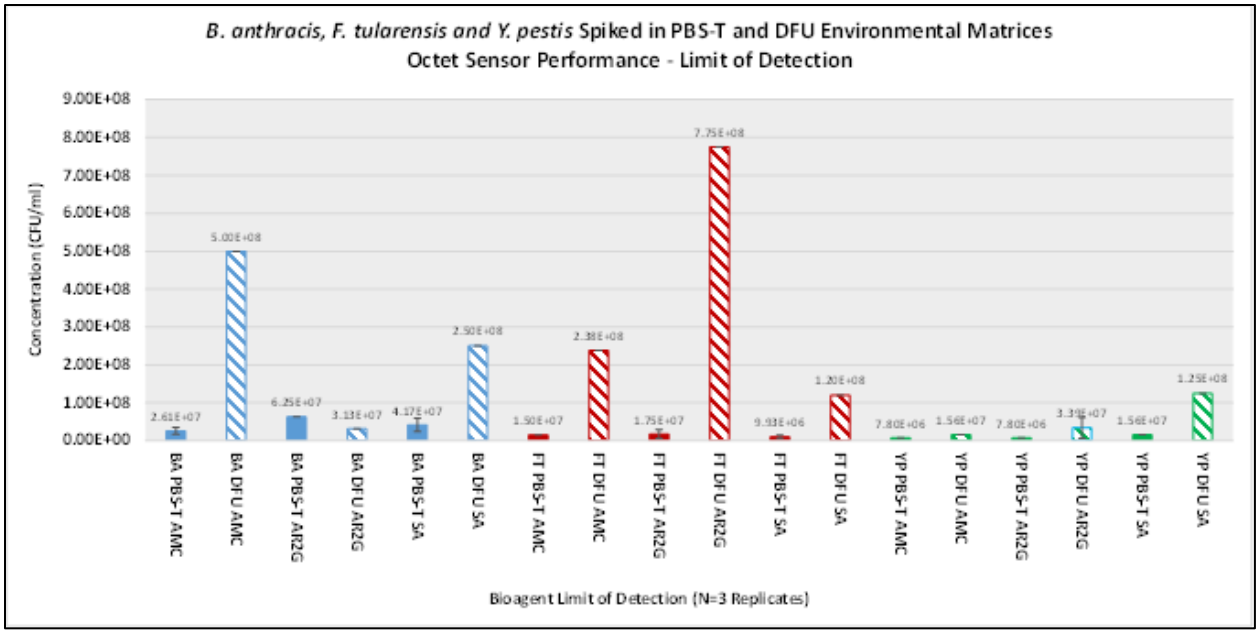

Figure 5. Octet sensor performance data for *B. anthracis, F. tularensis,* and *Y. pestis* pathogens spiked into PBS-T and DFU environmental matrices.

3.3 Task 3: TPR Determination for Octet Bioagent Assays

Using the downselected AR2G biosensor, a preliminary evaluation was conducted to determine the TPR, or sensitivity, for antigen cocktail mixtures in PBS-T and DFU environmental matrices (Table 11).

Table 11. Test Construct for Octet Sensitivity Evaluation

Cocktail Number	Bioagents	Buffer Matrix	DFU Matrix	Number of Tests per Bioagent					
				R	T2	BA	YP	FT	EB
1	R, T2, BA, EB	1	1	3	3	3			3
2	T2, BA, FT	1	1		3	3		3	
3	BA, FT, YP, R	1	1	3		3	3	3	
4	FT, YP, EB	1	1				3	3	3
5	YP, EB, R, T2	1	1	3	3		3		3
Total				9	9	9	9	9	9

R, RCA 120; T2, T2 mycotoxin; BA, *B. anthracis*; EB, Ebola VLPs; YP, *Y. pestis*; FT, *F. tularensis*.

Five antigen cocktail mixtures were prepared with analyte concentrations spiked into each respective cocktail at the assay cutoff level (as identified from LOD determinations). Each cocktail combination was run in triplicate with parallel reference sensors for subtracting the background. Included on the sensors were negative-control wells housing buffer and positive-control antigens at the LOD concentration specific to each antibody. The respective antibodies for each antigen in the cocktail were loaded onto the sensors using the same standard method that was applied during LOD determinations for the PBS-T and DFU evaluations (Tables 12 and 13, respectively).

Table 12. PBS-T Cocktail: Data Summary

PBS-T Cocktail Number	Antigen	Antigen Detection			TP	FN
		Replicate 1	Replicate 2	Replicate 3		
1	RCA 120	–	–	–	0	3
	T2 mycotoxin	+	+	+	3	0
	B. anthracis NNR-1	+	–	–	1	2
	Ebola VLPs	+	+	+	3	0
2	T2 mycotoxin	+	+	+	3	0
	B. anthracis NNR-1	+	+	+	3	0
	F. tularensis	+	+	–	2	1
3	*Y. pestis*	+	+	+	3	0
	F. tularensis	+	+	+	3	0
	B. anthracis NNR-1	+	+	+	3	0
	RCA 120	+	+	+	3	0
4	*Y. pestis*	+	+	+	3	0
	Ebola VLPs	+	+	+	3	0
	F. tularensis	+	+	+	3	0
5	*Y. pestis*	–	–	+	1	2
	Ebola VLPs	+	+	+	3	0
	RCA 120	+	+	+	3	0
	T2 mycotoxin	+	+	+	3	0

TP, true positive; FN, false negative; +, detected; –, not detected.

Table 13. DFU Cocktail: Data Summary

DFU Cocktail Number	Antigen	Antigen Detection			TP	FN
		Replicate 1	Replicate 2	Replicate 3		
1	RCA 120	–	–	+	1	2
	T2 mycotoxin	+	+	+	3	0
	B. anthracis NNR-1	–	–	–	0	3
	Ebola VLPs	–	–	–	0	3
2	T2 mycotoxin	+	+	+	3	0
	B. anthracis NNR-1	+	+	–	2	1
	F. tularensis	+	+	–	2	1
3	*Y. pestis*	+	+	+	3	0
	F. tularensis	–	–	–	0	3
	B. anthracis NNR-1	–	–	–	0	3
	RCA 120	–	–	–	0	3
4	*Y. pestis*	–	–	–	0	3
	Ebola VLPs	–	–	–	0	3
	F. tularensis	–	–	–	0	3
5	*Y. pestis*	+	+	+	3	0
	Ebola VLPs	+	+	+	3	0
	RCA 120	+	–	–	1	2
	T2 mycotoxin	+	+	–	2	1

TP, true positive; FN, false negative; +, detected; –, not detected.

Appendix B includes Octet sensograms that depict the wavelength shifts due to the biomolecular interactions that occurred when the antigen cocktail mix was spiked into either the PBS-T- or the DFU-extracted matrix. Given the assumption of generating a true-positive (TP) and/or a false-negative (FN) response, the equation used to calculate TPR for the evaluations was as follows:

$$TPR = TP/(TP + FN)$$

Table 14 describes the preliminary TPR determinations for both cocktailed samples in PBS-T and DFU matrices at the LOD concentrations. Bioagents spiked at their respective LODs in PBS-T buffer yielded sensitivity TPRs ranging from 67% (for RCA 120) to 100% (for T2 mycotoxin and Ebola VLPs). Concurrently, for the exception of the T2 mycotoxin assays, the TPRs for the bioagents spiked in DFU matrix were significantly lower (by 22–33%).

Table 14. TPR (Sensitivity)

Bioagent	PBS-T		DFU	
	FN/TP	TPR (%)	FN/TP	TPR (%)
RCA 120	3/6	67	7/2	22
T2 mycotoxin	0/9	100	1/8	89
B. anthracis	2/7	78	7/2	22
Y. pestis	2/7	78	6/3	33
F. tularensis	1/8	89	7/2	22
Ebola VLPs	0/9	100	6/3	33

4. DISCUSSION AND CONCLUSIONS

The Octet platform was selected to examine the feasibility for conducting high-throughput analysis for environmental sampling. The process involved using label-free BLI technology to probe the biomolecular interactions between DBPAO monoclonal antibodies and their respective analytes in a 96- and 384-well format. During the preliminary evaluation, antibodies for *B. anthracis*, *F. tularensis*, *Y. pestis*, Ebola VLPs, T2 mycotoxin, and RCA 120 were immobilized onto Octet SA, AMC, and AR2G sensor tips using Dip and Read kit applications to anchor the proteins to the sensor. The LOD was determined for each bioagent spiked into both PBS-T- and DFU-extracted milieus on all three sensor types.

The results generated in Task 1 were compiled and statistically weighted to support identification of the best overall sensor to carry forward to sensitivity testing in Task 3, based on the relationship between the LOD values and SDs for the PBS-T and DFU matrices. The results for bioagents spiked in the DFU-extracted matrix were given more weight than those in PBS-T for the sensor downselection to better represent biological samples extracted from DFU matrices. All three sensors performed identically for the T2 mycotoxin assay; LOD cutoffs were 500 µg/mL and the CV was 0%. The AR2G sensor outperformed the AMC and SA sensors: the *B. anthracis* LOD was 3.13E+07 cfu/mL (CV of 0%) for the AR2G sensor, compared with 5.00E+08 and 2.50E+08 cfu/mL for the AMC and SA sensors, respectively. The SA sensor performed well for the small-molecule RCA 120 and T2 mycotoxin LOD assays. In the end, the

AR2G sensor was downselected for the Task 3 sensitivity testing as a result of its overall consistency in the large-molecule assays (particularly *B. anthracis* NNR-1 spores) combined with the fact that antibody coupling for the AR2G sensor required only one step, whereas the SA sensor preparation required two steps.

The data suggest that the Octet responses for the cocktailed analyte samples (spiked in PBS-T and DFU matrices at the LODs) demonstrated limitations when the samples were in the presence of other competing organisms. This was particularly true when the DFU matrix was used. The PBS-T samples yielded sensitivity TPRs of 67% (for RCA 120), 78% (for *B. anthracis* and *Y. pestis*), 89% (for *F. tularensis*), and 100% (for T2 mycotoxin and Ebola VLPs). In contrast, the TPRs for the bioagent cocktails spiked in DFU matrix were significantly (22–33%) lower, with the exception of the T2 mycotoxin assays (89%). Due to the complex qualities of the DFU-extracted medium, further optimization of the bioassays would be desirable. This would be followed by more extensive testing to determine new LODs in the presence of competing organisms, which displayed poorly in the sensitivity testing. Although the AR2G sensor was selected for the follow-on TPR sensitivity testing, the initial LOD results predicated the need for a deeper look into more extensive optimization of the assays, particularly for the DFU matrix. The conclusion that "one size does not fit all" is probable and applies to future efforts dedicated to establish more sensitive LOD cutoffs for the bioagent assays. The observation that the SA sensor appeared to be more suited for small-molecule assays, whereas the AMC and AR2G sensors seemed better paired for large-molecule detection, is a subject for further experimentation. Using this supposition, a logical next step would be to focus on optimizing individual antigen assays (particularly whole organisms) with specific sensors, to achieve more sensitive assays for DFU matrices.

The initial scope of this effort included testing to assess not only false-negative evaluations, but also false-positive responses for the cocktailed mixtures. However, it became clear after the TPR testing that further assay optimization is necessary to move in this direction because of issues experienced with the DFU matrix and low TPRs. During the evaluation, a few unexpected issues and insights were noted that relate to certain antigens. For instance, T2 mycotoxin is supposedly only soluble in chloroform, and this presented a difficult hurdle to overcome. The Octet sensors were stripped in the presence of chloroform; therefore, attempts were made to identify a buffer that did not destroy the sensors, yet preserved the functionality of the T2 mycotoxin, namely, PBS-T with 10% dimethyl sulfoxide and 0.5% bovine serum albumin. Another notable issue was that large molecules (i.e., whole organisms) had a tendency to clump, invert the sensograms, and nonspecifically bind. In these instances, the data became difficult to interpret, and recognizing the trends and patterns in the sensograms helped not only to interpret but also possibly to eliminate the issues.

Particularly interesting during this effort was the ability to purchase commercially available reagent coupling kits from manufacturers to support the performance of in-house method development (as opposed to contractual outsourcing) and to immobilize DBPAO proteins of interest to the various biosensor surfaces using the simplified coupling chemistries from the Dip and Read kit applications. Having an in-house capability to produce tailor-made, high-throughput biosensor microplates at a significant cost reduction to enable environmental sampling scenarios was an important consideration during this evaluation. Toward this end,

efforts were made to (1) gauge the "methods and materials" processes to help factor in a ROM estimate of the relative cost per assay for reagents, (2) ascertain the potential for method-friendly manipulations for in-house development of the sensors, and (3) recognize the level of skill required by laboratory personnel to manufacture the sensors in-house. This capability is desirable in that it allows for mission-specific "designer" microplate sensors to be customized in-house, thereby precluding the delays and contractual interruptions that often occur with outsourcing of materials. The approximate time frame required to perform antibody coupling to the sensors is dependent upon the purity of the antibody and nuances of the coupling chemistry, as well as the analyst's familiarity with the laboratory skills and techniques required to perform such immobilizations. The DBPAO antibodies have long been used by the authors for assay development, which provided a historical baseline for handling the antibodies for this effort. At a minimum, antibody coupling for the SA and AMC kits took approximately 1 h. All antibodies used for the AR2G sensor coupling were first dialyzed into PBS-T overnight using a standard dialysis cassette. Three buffer exchanges were performed (one per day) to ensure removal of amine-containing contaminants such as Tris, ammonium sulfate, and others. Because the AR2G sensors required an amine-coupling reaction, the buffer used to dilute the DBPAO monoclonal antibodies for loading had to be at least 1 to 1.5 points higher than the pH of the buffer used for immobilization. For this study, the EDC/sulfo-NHS concentration remained constant, and the DBPAO monoclonal antibodies loaded optimally in an immobilization buffer of 10 mM sodium acetate with pH 5. If the isoelectric point of an antibody to be used in amine coupling is not known, then this step may take longer to optimize an immobilization buffer and/or an activation buffer and could require 3–4 h.

Another important task during this evaluation was to approximate the general cost per assay (reagents and kits only, with labor excluded) for in-house manufacture of the sensor microplates. Generally, for this effort, one assay consisted of 8 concentrations in a titration series with a parallel reference column; therefore, 16 total sensors were required at roughly $5 per sensor or $80 total. Additional assay costs included kinetics buffer ($50 per bottle), which is equivalent to approximately $1 per assay, a reagent plate ($12), and a sensor soaking plate ($5). The costs involved in purchasing the commercial coupling kits were relatively moderate when compared to those associated with outsourcing. The amine coupling kits for the AR2G assay were $857 and contained enough material for 960 reactions ($1 per reaction or $16 per assay). Overall, to perform a standard titration series, the costs were roughly $114 for AR2G and $98 for SA and AMC. The cost to run samples on an entire microplate housing 96 total reactions was $684 for AR2G and $588 for SA and AMC. These estimates do not include the costs of antibodies, antigens, and positive-control reagents.

One-time costs associated with the Octet platform operation, maintenance contracts, and ancillary equipment and software are as follows:

- service contract for Octet Red384 system: $30,000;
- service contract for HTX system: $40,000;
- preventive maintenance outside of service contract: $6000–9000;
- biosensor dispenser: $642;
- mount cleaning trays (one-time use, pack of 12): $120; and
- Octet CFR software: $10,710.

Overall, the Octet system enabled high-throughput sample analysis with fewer manipulations and provided a comprehensive data output. The preliminary data suggest that the Octet platform has the potential for use as a sample analysis platform. The scope of this effort did not include optimization and fine-tuning of the bioagent assays to a specific sensor; nonetheless, the LOD and TPR values observed for the samples spiked in PBS-T established a baseline that can be used for further optimization. Although the BLI method requires little to no sample processing, the Octet system demonstrated limitations in the presence of other competing organisms for DFU matrices in this testing scenario.

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2. ForteBio Biologics by Molecular Devices. Biosensors and Kits. https://www.moleculardevices.com/products/biologics/accessories-consumables/biosensors-kits (accessed 9 July 2019).

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ACRONYMS AND ABBREVIATIONS

AMC	anti-mouse Fc capture
AR2G	amine reactive second generation
BA	*Bacillus anthracis* NNR-1 spores
BLI	biolayer interferometry
CCDC CBC	U.S. Army Combat Capabilities Command Chemical Biological Center
cfu	colony-forming units
CV	coefficient of variation
DBPAO	Defense Biological Product Assurance Office
DFU	dry filter unit
EDC	1-ethyl-3-(3-(dimethylamino) propyl carbodiimide
FN	false negative
FT	*Francisella tularensis* var. *tularensis*
K_D	equilibrium dissociation constant
LOD	limit of detection
Mab	monoclonal antibody
PBS-T	phosphate-buffered saline with 0.1% Triton X-100
RCA 120	*Ricinus communis* agglutinin
ROM	rough order of magnitude
SA	streptavidin
SD	standard deviation
sulfo-NHS	sulfo-*N*-hydroxysuccinimide
T2	T2 mycotoxin
TP	true positive
TPR	true positive rate
VLP	virus-like particle
YP	*Yersinia pestis* EV76

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APPENDIX A

OCTET SENSOGRAM RESULTS FOR ENVIRONMENTAL MATRICES TESTING USING PHOSPHATE-BUFFERED SALINE WITH 0.1% TRITON X-100 (PBS-T) AND DRY FILTER UNIT (DFU)

A.1 Anti-mouse Fc Capture (AMC) Sensors: *Yersinia pestis* EV76 in PBS-T

- *Y. pestis* EV76 antigen was diluted in a twofold titration series ranging from 2.50E+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 7.80E+06 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

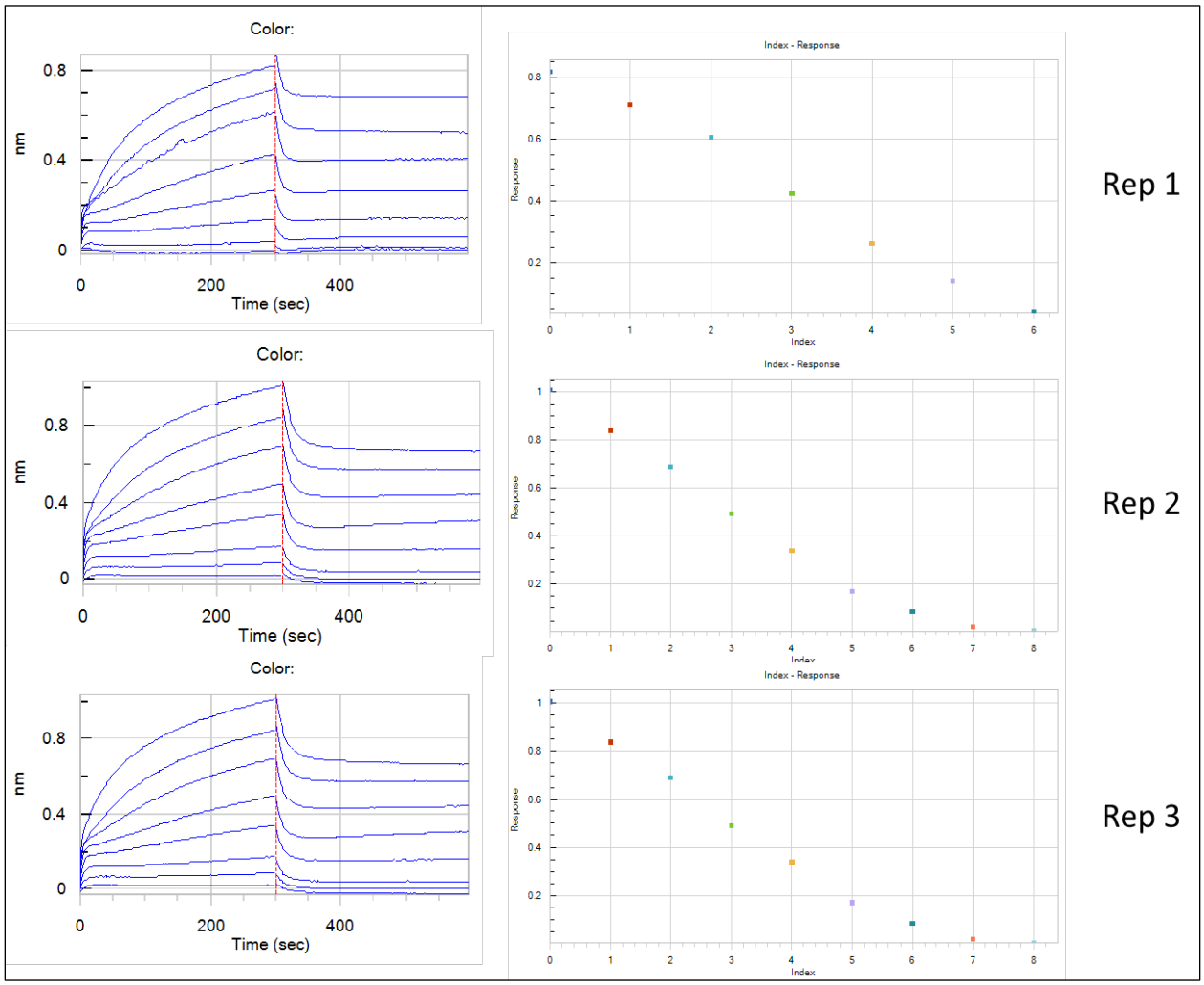

Figure A1. AMC sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* monoclonal antibody (Mab 1) in PBS-T, replicates (Rep) 1–3.

A.2 AMC Sensors: *Y. pestis* EV76 in DFU Matrix

- *Y. pestis* EV76 antigen was diluted in a twofold titration series ranging from 2.50E+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 1.56E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

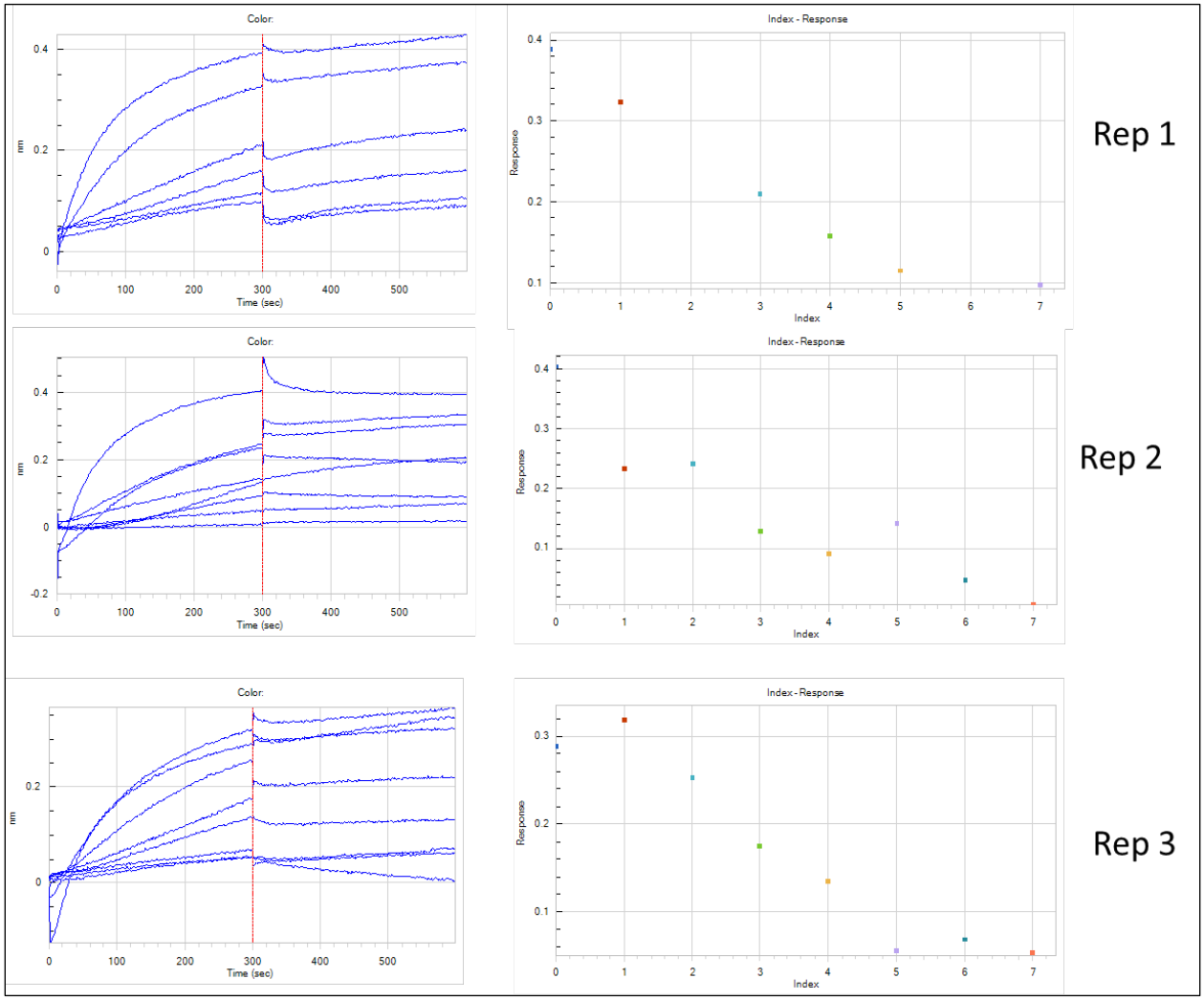

Figure A2. AMC sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* Mab 1 in DFU matrix, Rep 1–3.

A.3 Streptavidin (SA) Sensors: *Y. pestis* EV76 in PBS-T

- *Y. pestis* EV76 antigen was diluted in a twofold titration series ranging from 2.50E+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 1.56E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

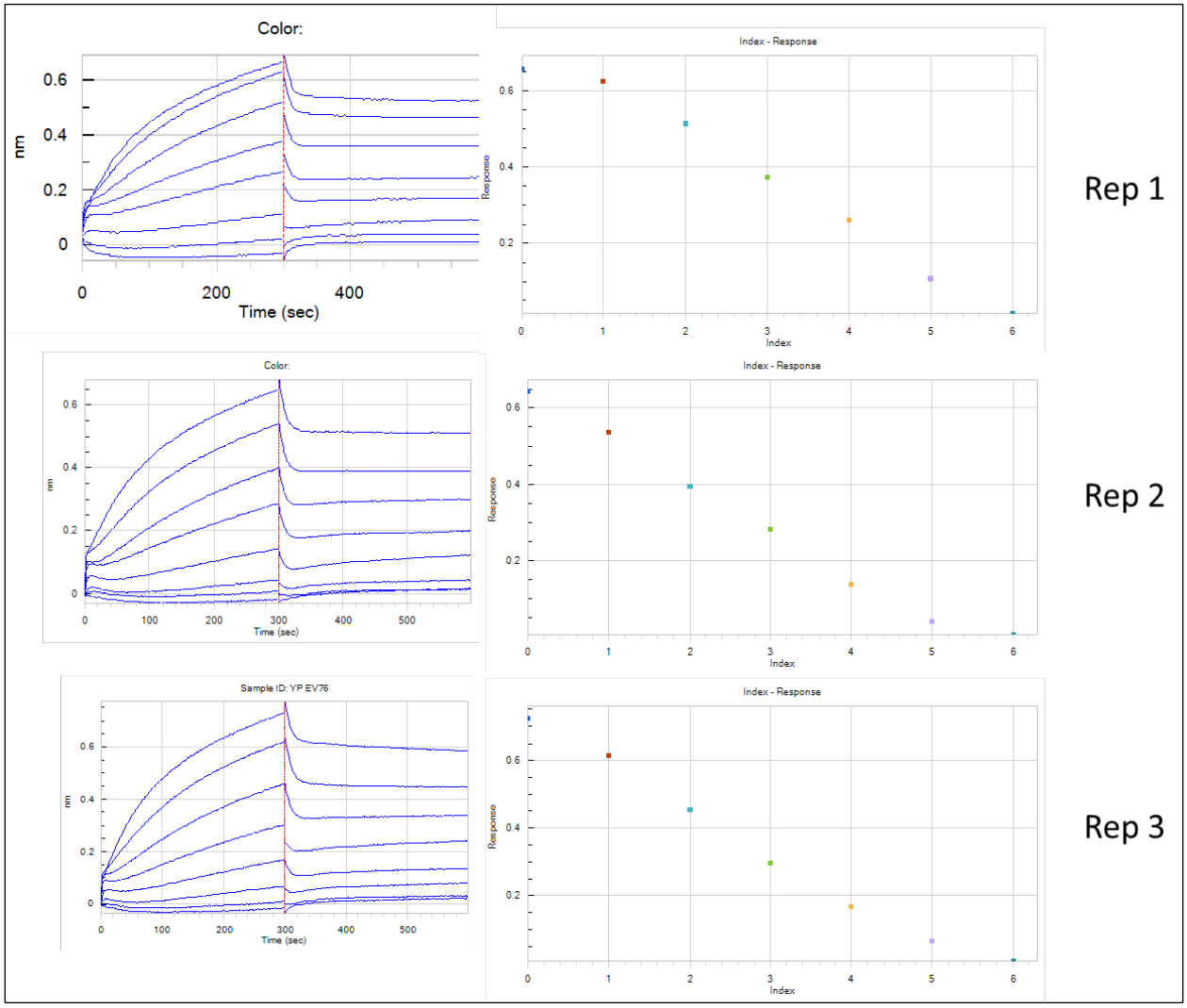

Figure A3. SA sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* Mab 1 in PBS-T, Rep 1–3.

A.4 SA Sensors: *Y. pestis* EV76 in DFU Matrix

- *Y. pestis* EV76 antigen was diluted in a twofold titration series ranging from 2.50E+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 1.25E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

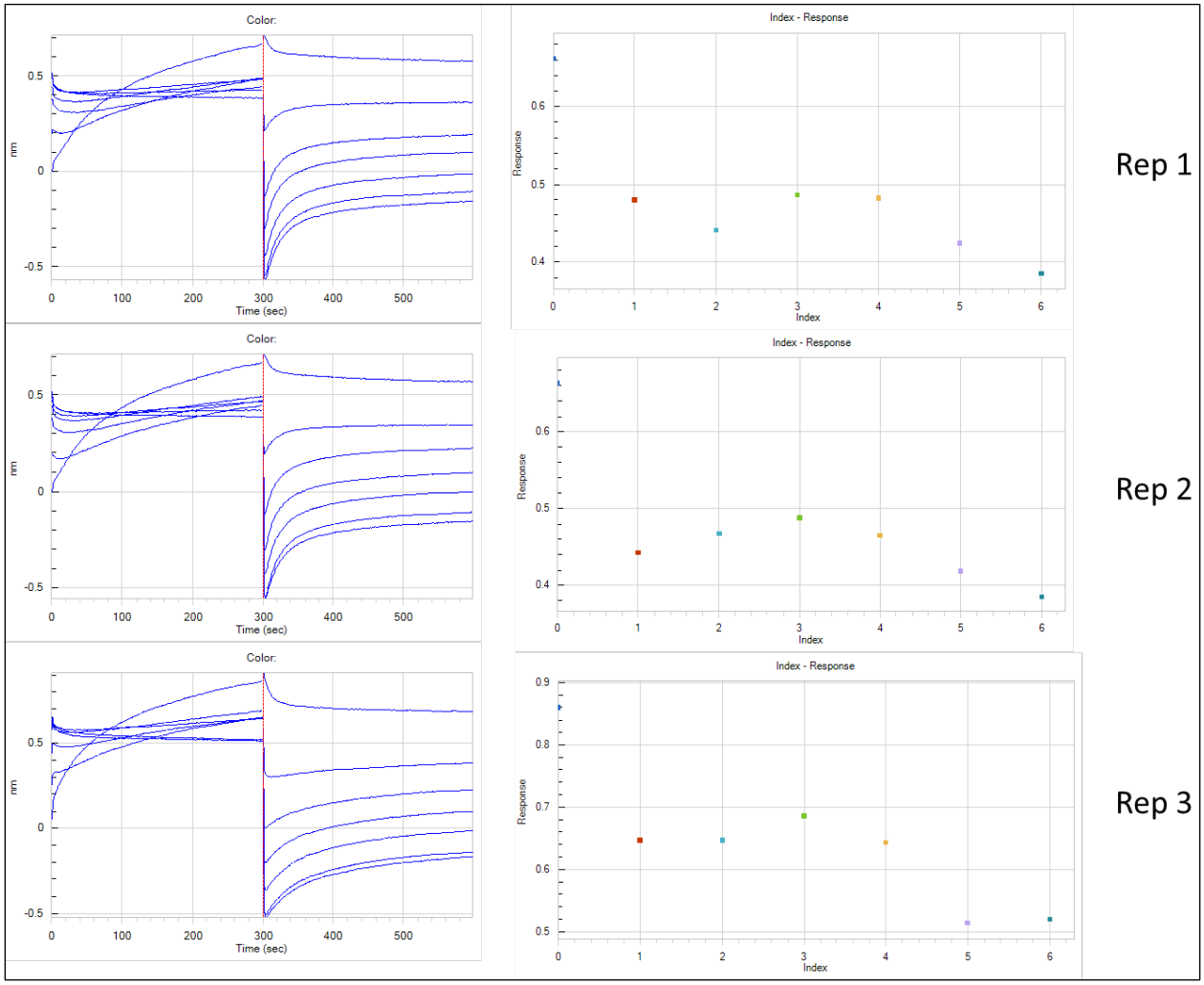

Figure A4. SA sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* Mab 1 in DFU matrix, Rep 1–3.

A.5 Amine Reactive Second Generation (AR2G) Sensors: *Y. pestis* EV76 in PBS-T

- *Y. pestis* EV76 antigen was diluted in a twofold titration ranging from 2.50E+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 7.80E+06 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

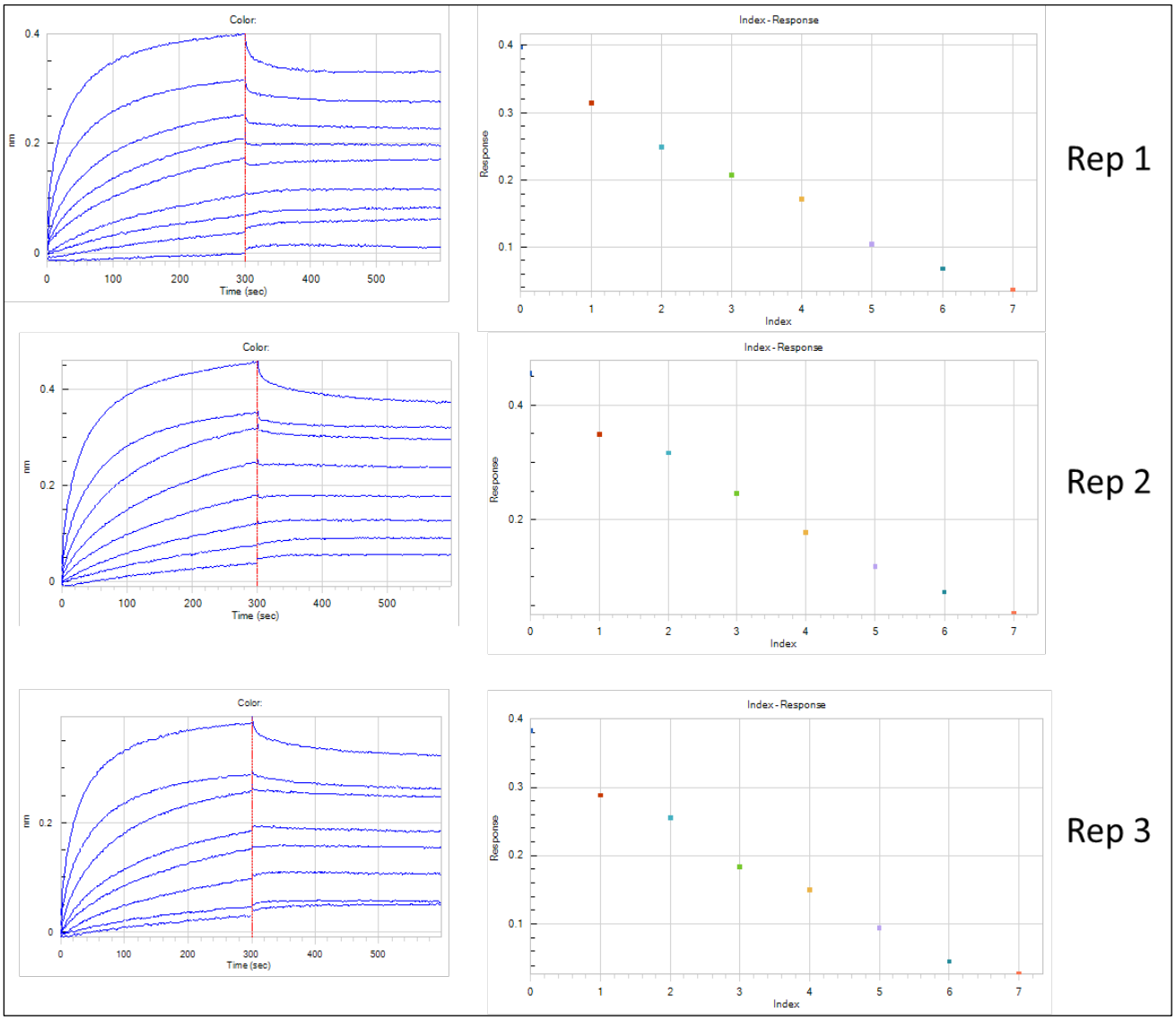

Figure A5. AR2G sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* Mab 1 in PBS-T, Rep 1–3.

A.6 AR2G Sensors: *Y. pestis* EV76 in DFU Matrix

- *Y. pestis* EV 76 antigen was diluted in a twofold titration series ranging from 2.50+08 to 1.50E+04 cfu/mL.
- *Y. pestis* EV76 antigen was detected down to 3.13E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

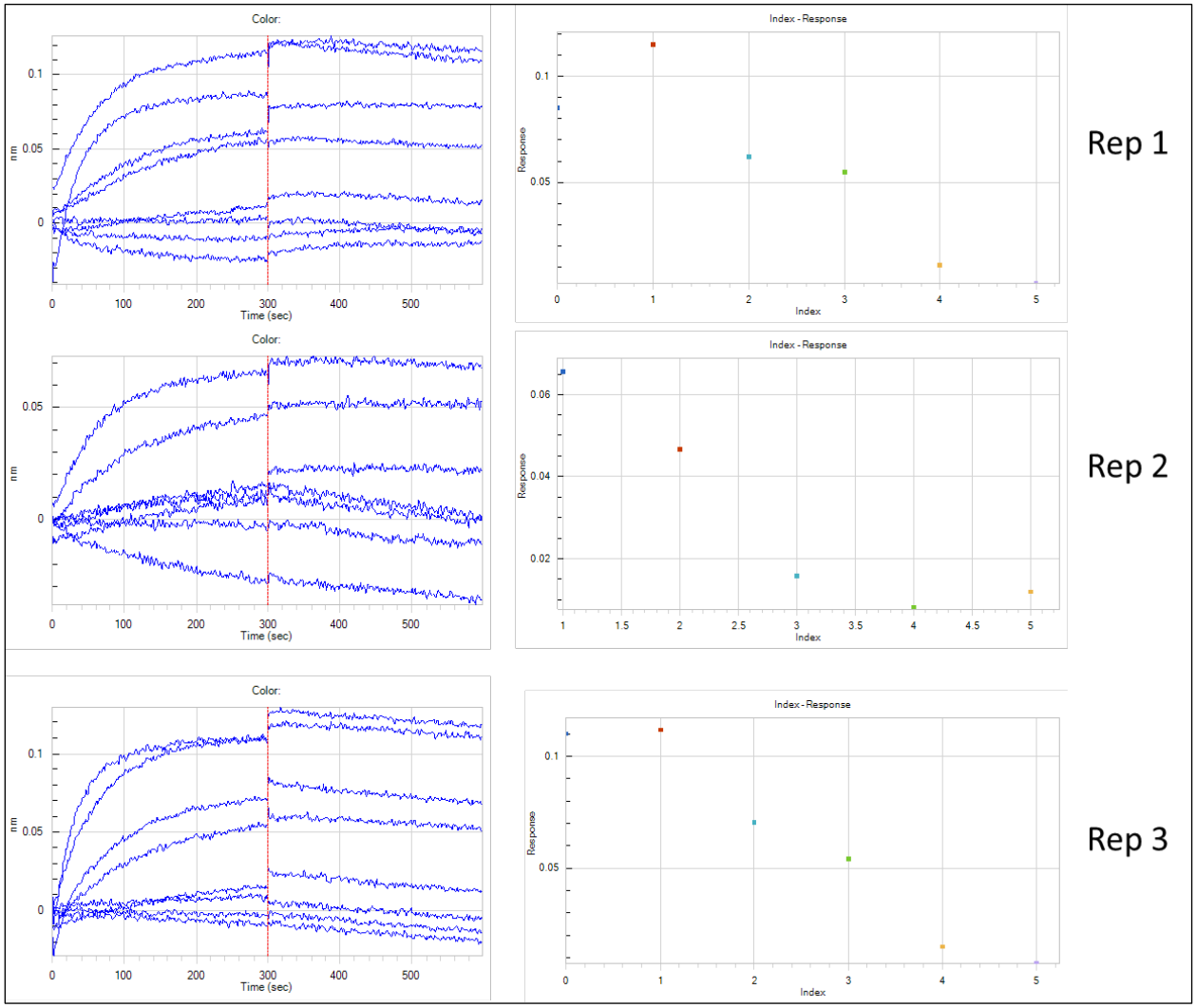

Figure A6. AR2G sensograms for *Y. pestis* EV76 antigen with anti-*Y. pestis* Mab 1 in DFU matrix, Rep 1–3.

A.7 AMC Sensors: *Francisella tularensis* in PBS-T

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* antigen was detected down to 1.50E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

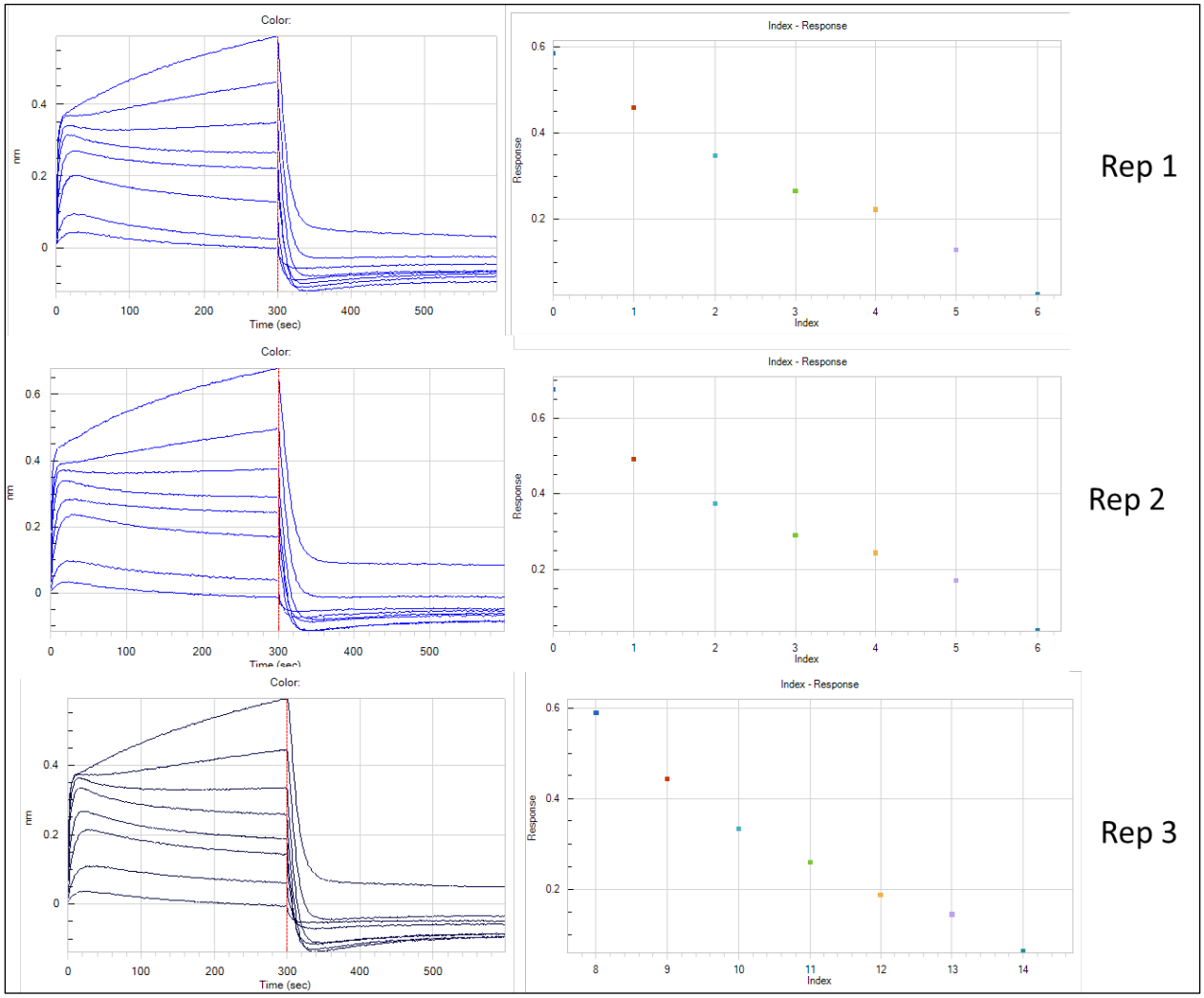

Figure A7. AMC sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in PBS-T, Rep 1–3.

A.8 AMC Sensors: *F. tularensis* in DFU Matrix

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* antigen was detected down to 2.38E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

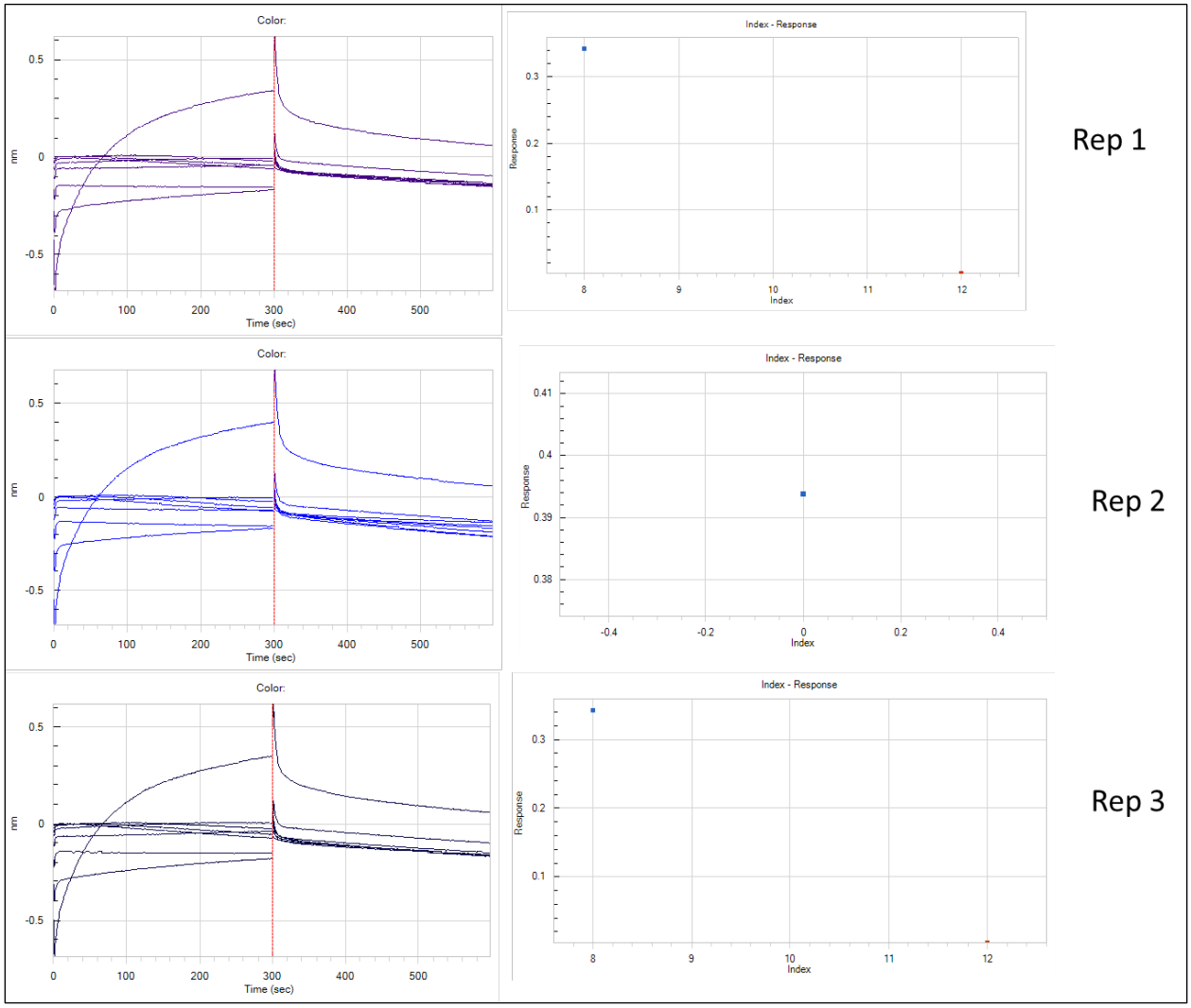

Figure A8. AMC sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in DFU matrix, Rep 1–3.

A.9 AR2G Sensors: *F. tularensis* in PBS-T

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* antigen was detected down to 1.50E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

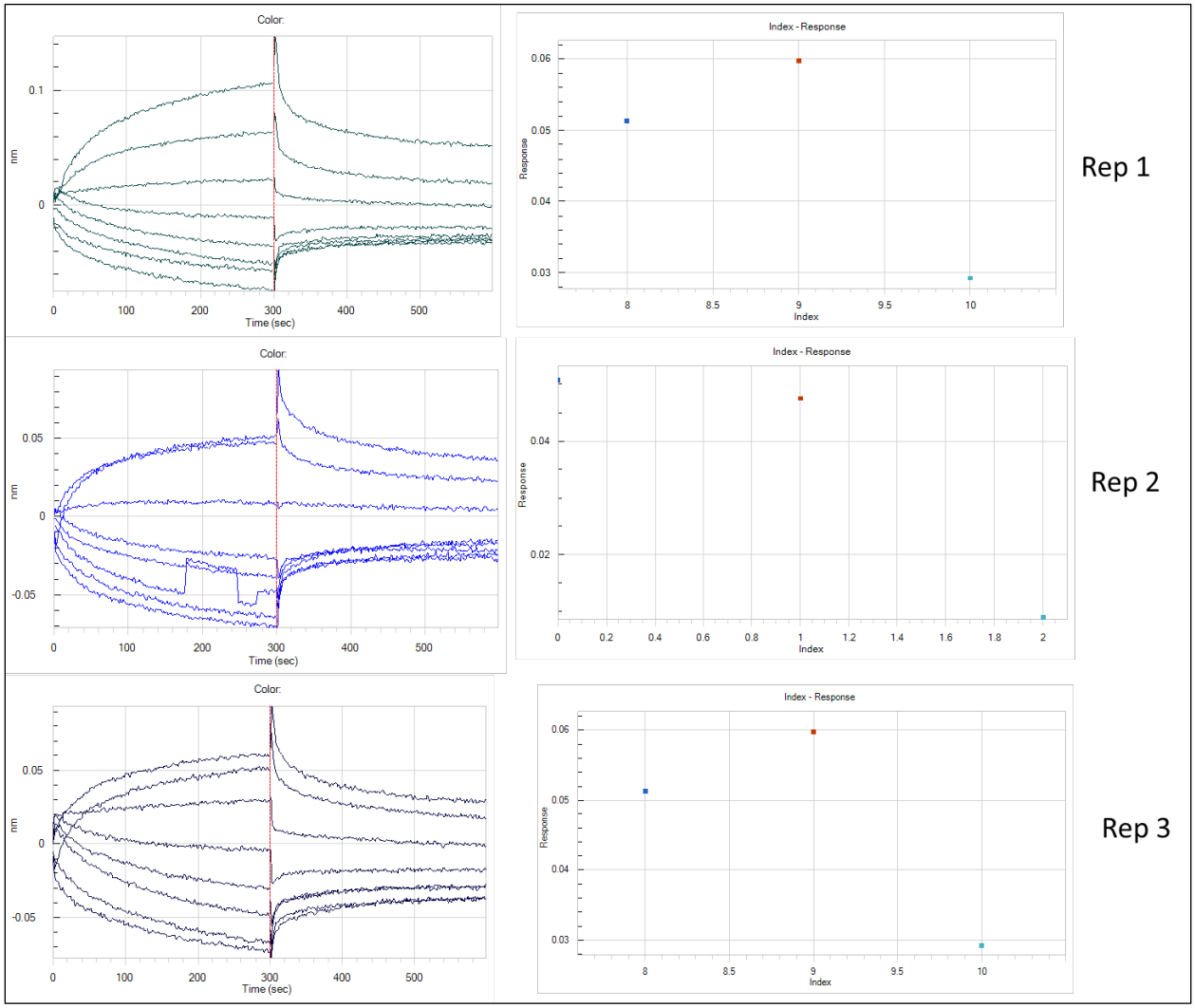

Figure A9. AR2G sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in PBS-T, Rep 1–3.

A.10 AR2G Sensors: *F. tularensis* in DFU Matrix

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* antigen was detected down to 4.75E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

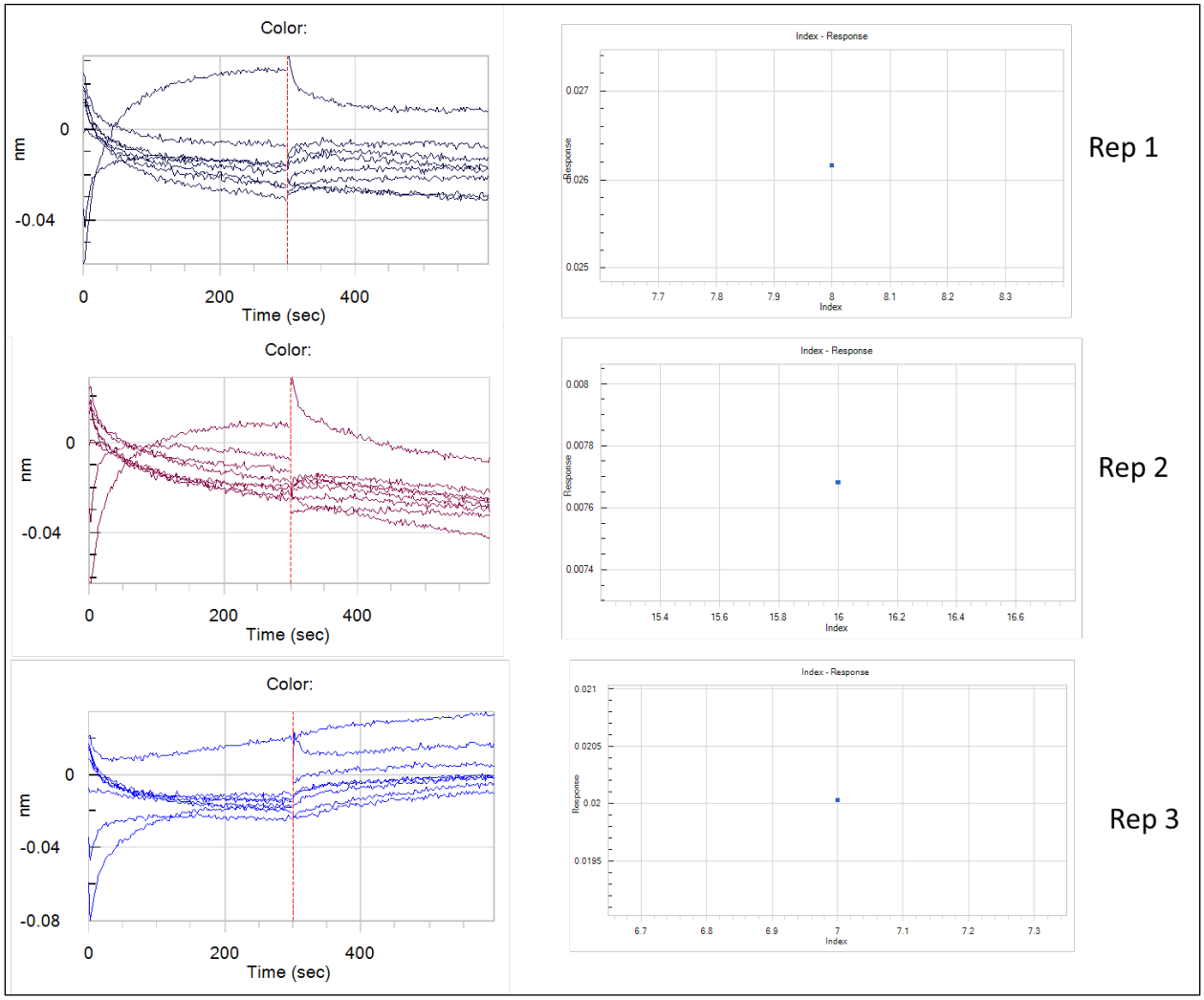

Figure A10. AR2G sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in DFU matrix, Rep 1–3.

A.11 SA Sensors: *F. tularensis* in PBS-T

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* antigen was detected down to 7.40E+06 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

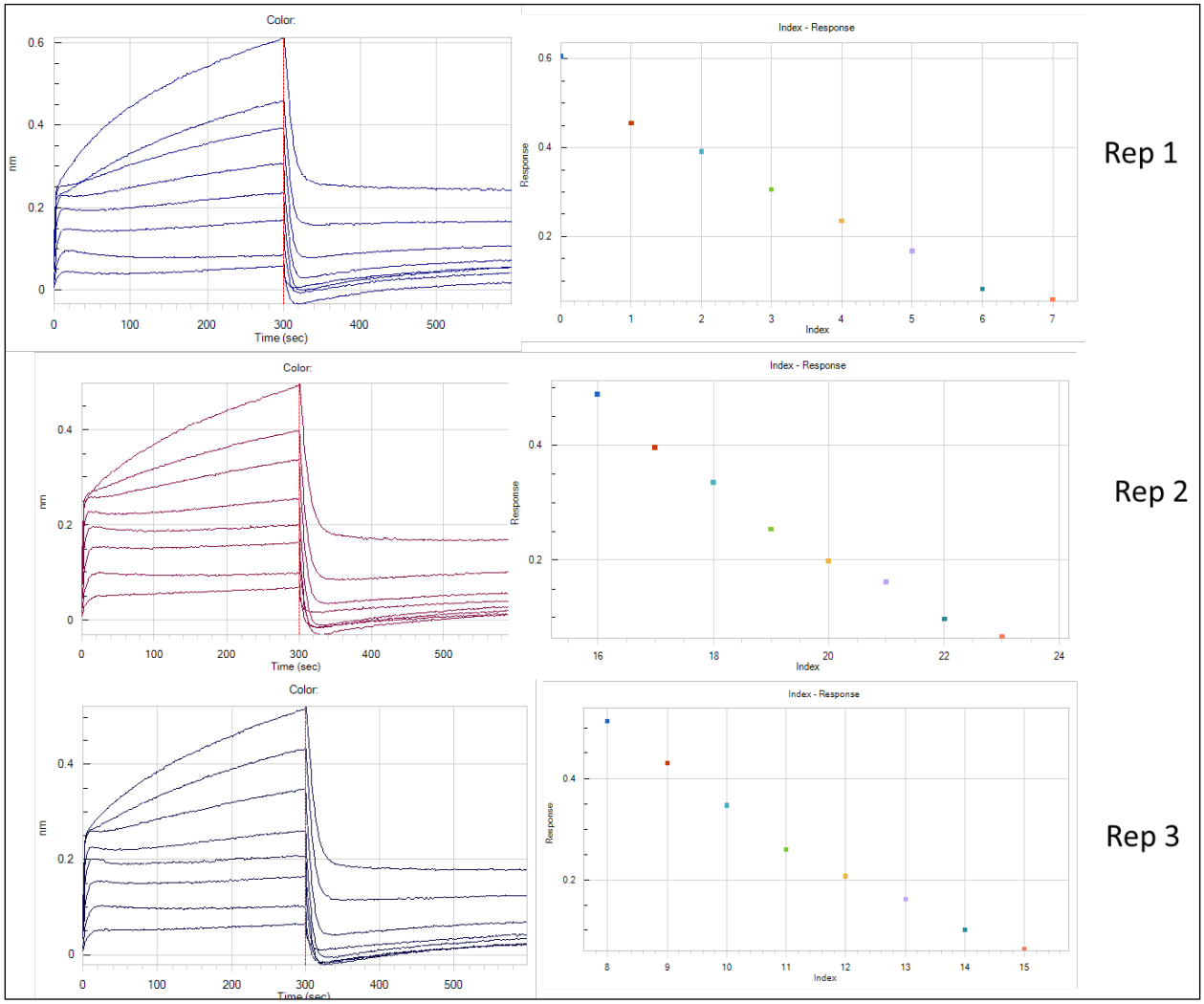

Figure A11. SA sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in PBS-T, Rep 1–3.

A.12 SA Sensors: *F. tularensis* in DFU Matrix

- *F. tularensis* antigen was diluted in a twofold titration series ranging from 4.50E+08 to 9.25E+05 cfu/mL.
- *F. tularensis* was detected down to 1.28E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

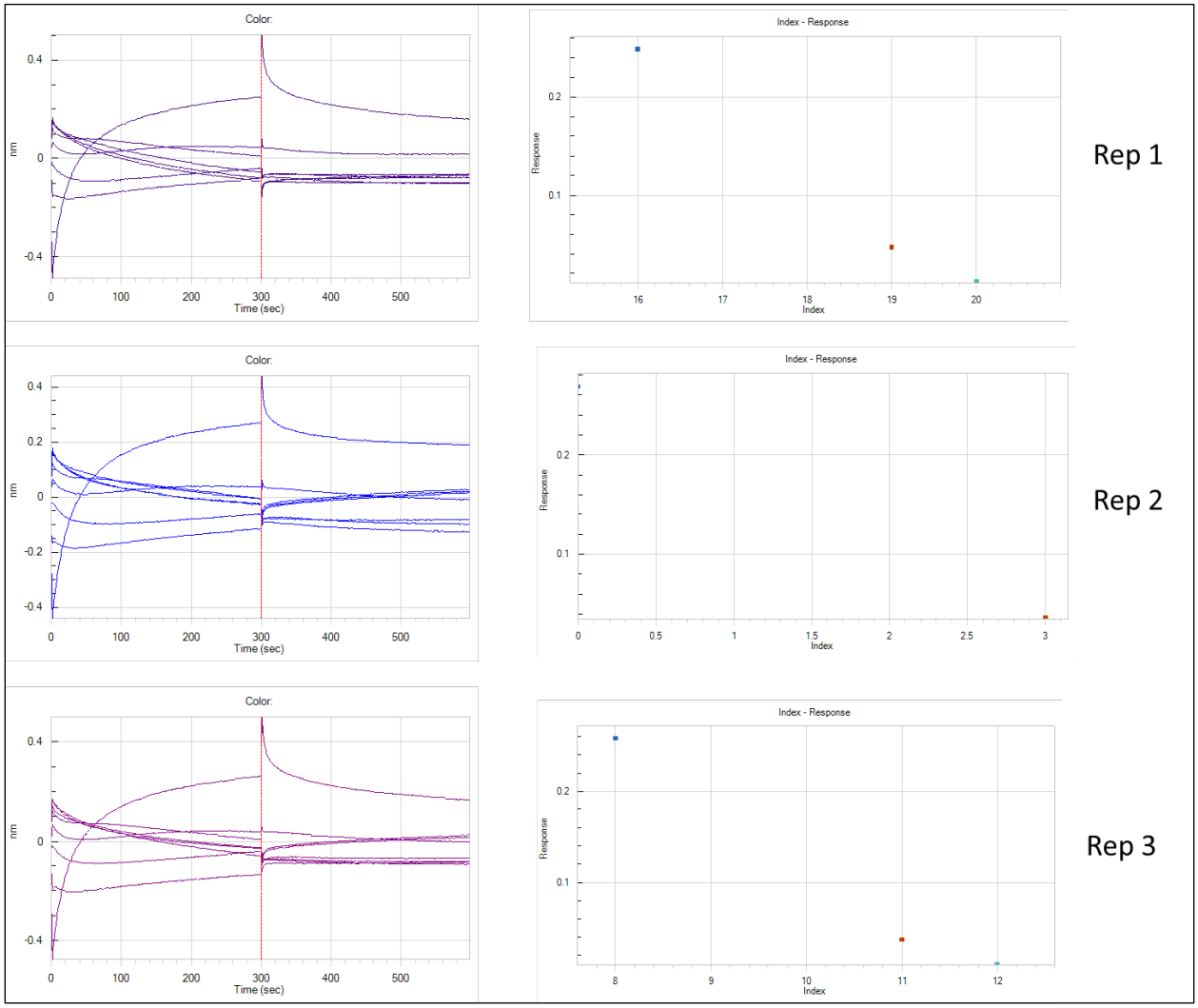

Figure A12. SA sensograms for *F. tularensis* antigen with anti-*F. tularensis* Mab 2 in DFU matrix, Rep 1–3.

A.13 AMC Sensors: *Ricinus communis* agglutinin (RCA 120) in PBS-T

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 238 pg/mL.
- RCA 120 was detected down to 3.81 ng/mL.
- Equilibrium dissociation constant (K_D) was 5.74 nM.

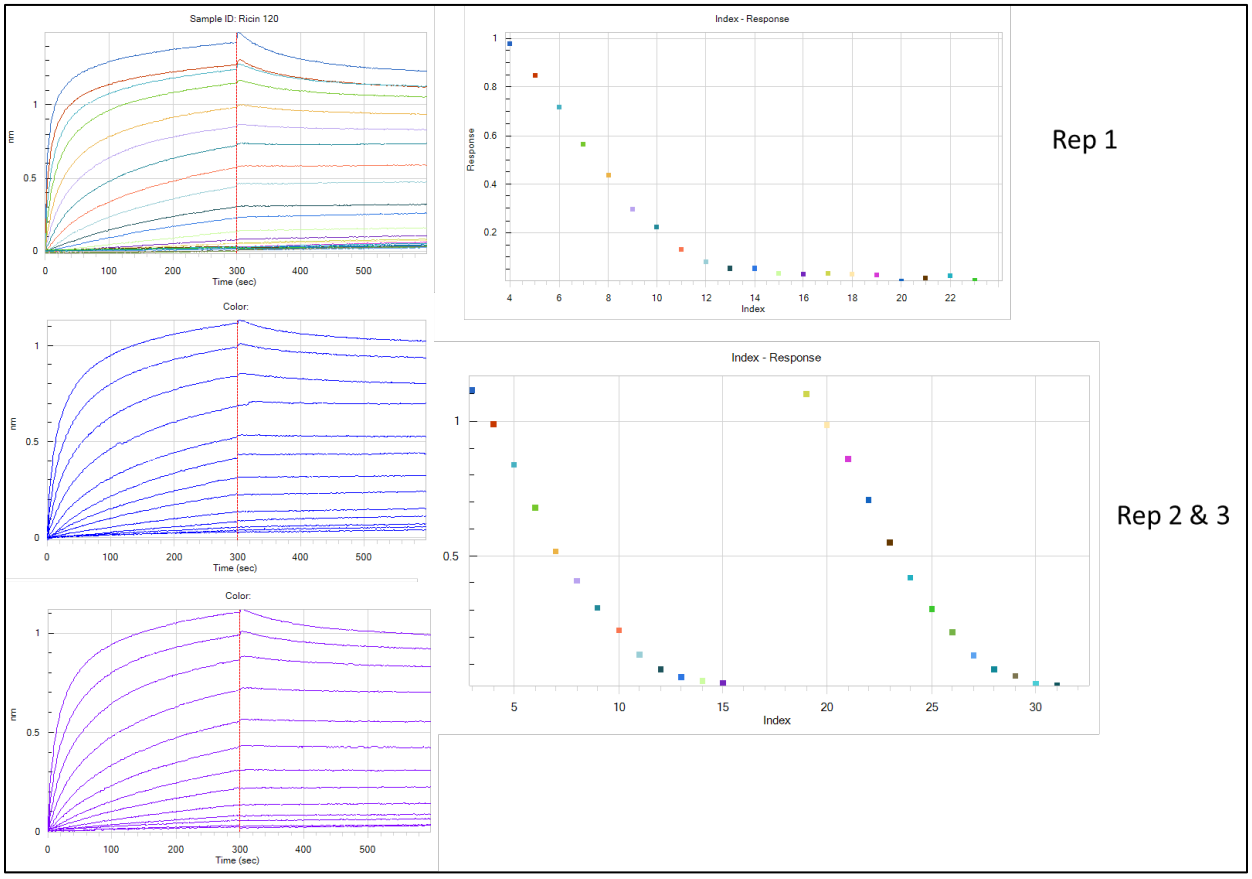

Figure A13. AMC sensograms for RCA 120 with anti-ricin Mab 1 in PBS-T, Rep 1–3.

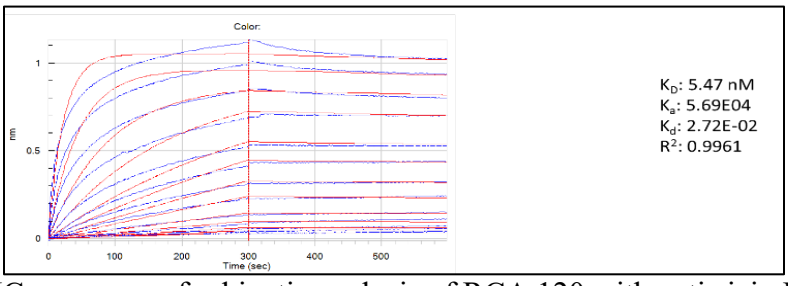

Figure A14. AMC sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in PBS-T.

A.14 AMC Sensors: RCA 120 in DFU Matrix

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 238 pg/mL.
- RCA 120 was detected down to 1.91 ng/mL.
- K_D was 7.87 nM.

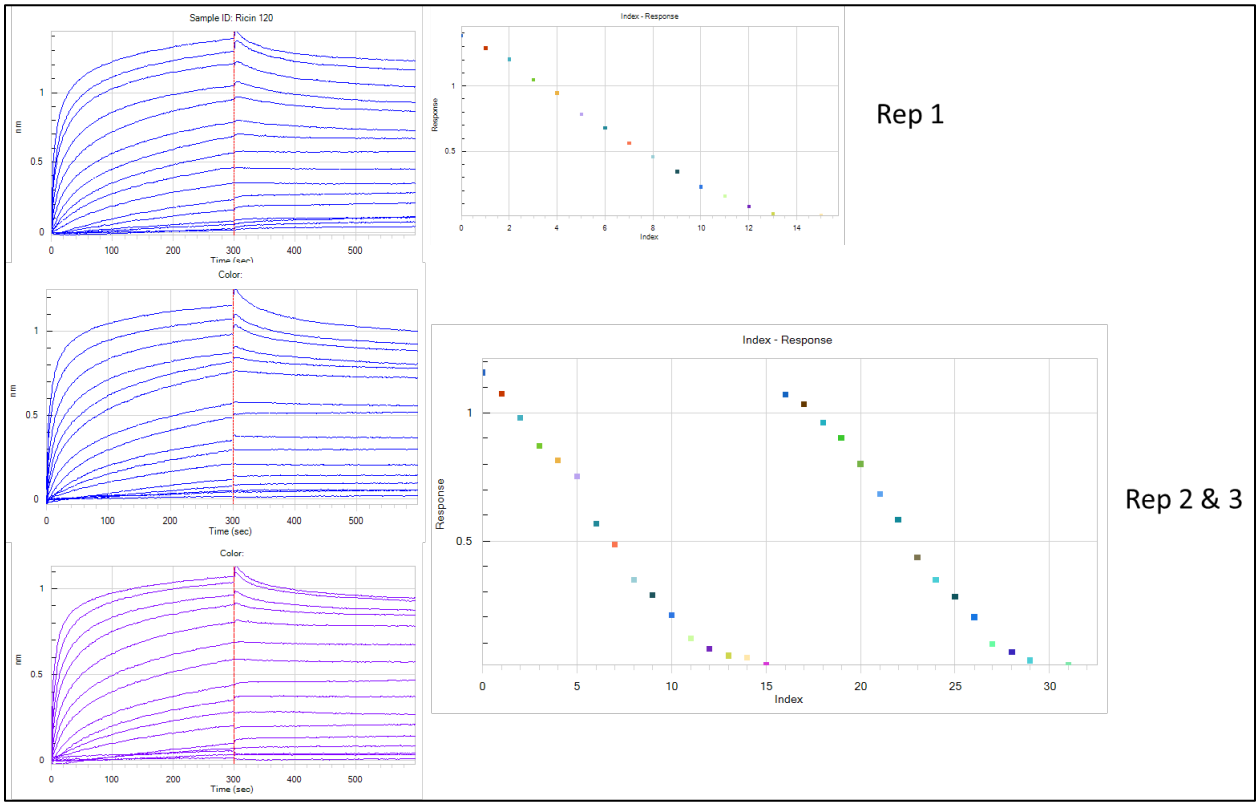

Figure A15. AMC sensograms for RCA 120 with anti-ricin Mab 1 in DFU matrix, Rep 1–3.

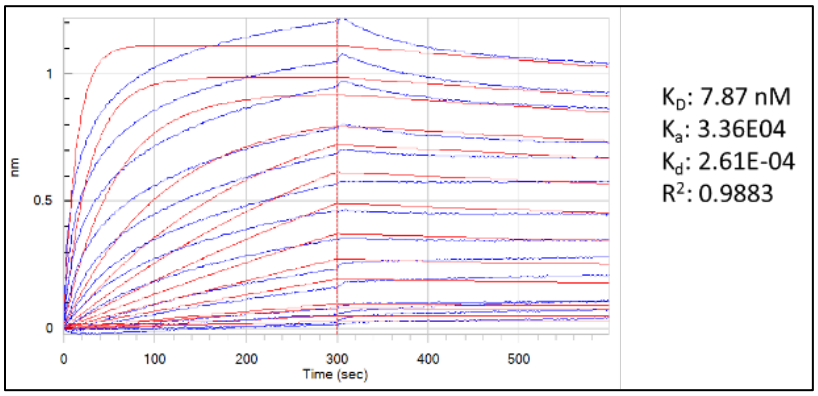

Figure A16. AMC sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in DFU matrix.

A.15 SA Sensors: RCA 120 in PBS-T

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 238 pg/mL.
- Ricin was detected down to 953 pg/mL.
- K_D was 1.82 nM.

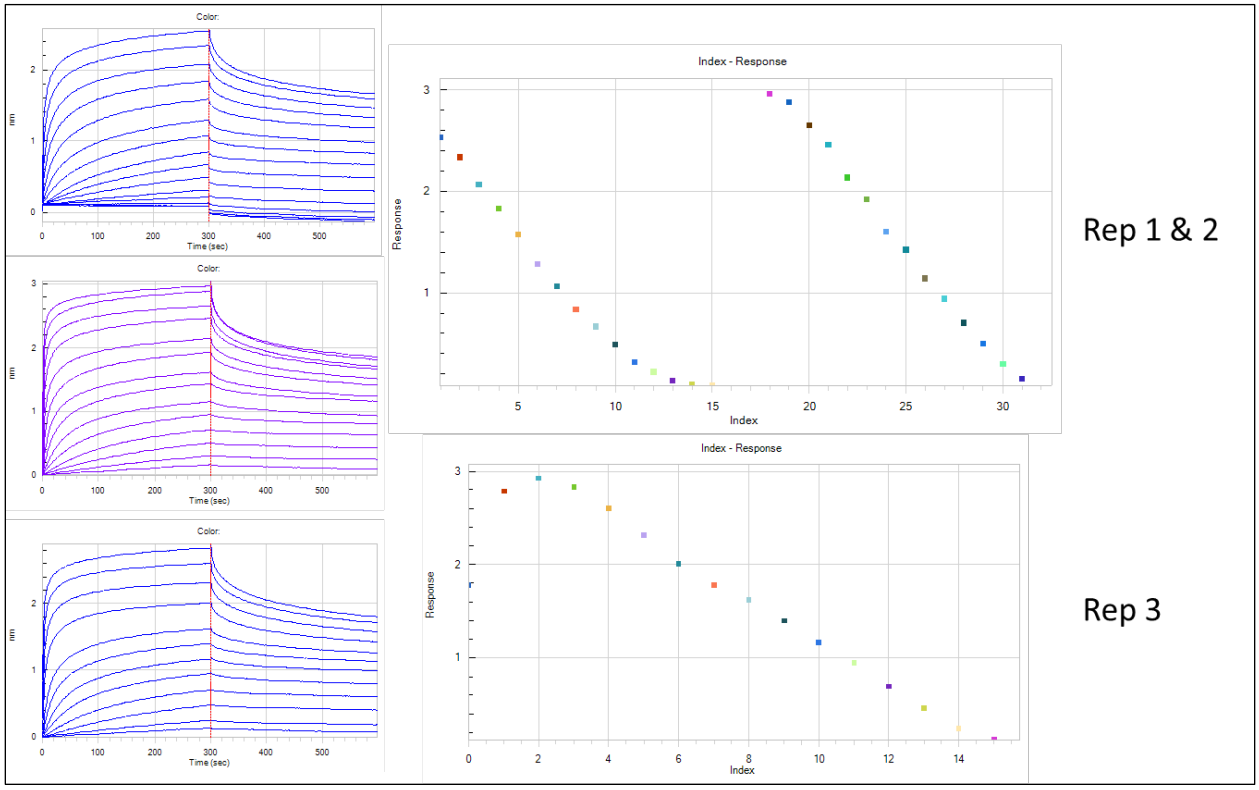

Figure A17. SA sensograms for RCA 120 with anti-ricin Mab 1 in PBS-T, Rep 1–3.

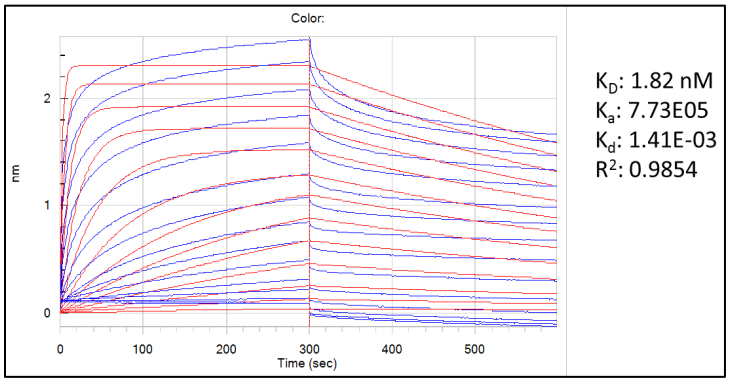

Figure A18. SA sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in PBS-T.

A.16 SA Sensors: RCA 120 in DFU Matrix

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 234 pg/mL.
- RCA 120 was detected down to 953 pg/mL.
- K_D was 17 nM.

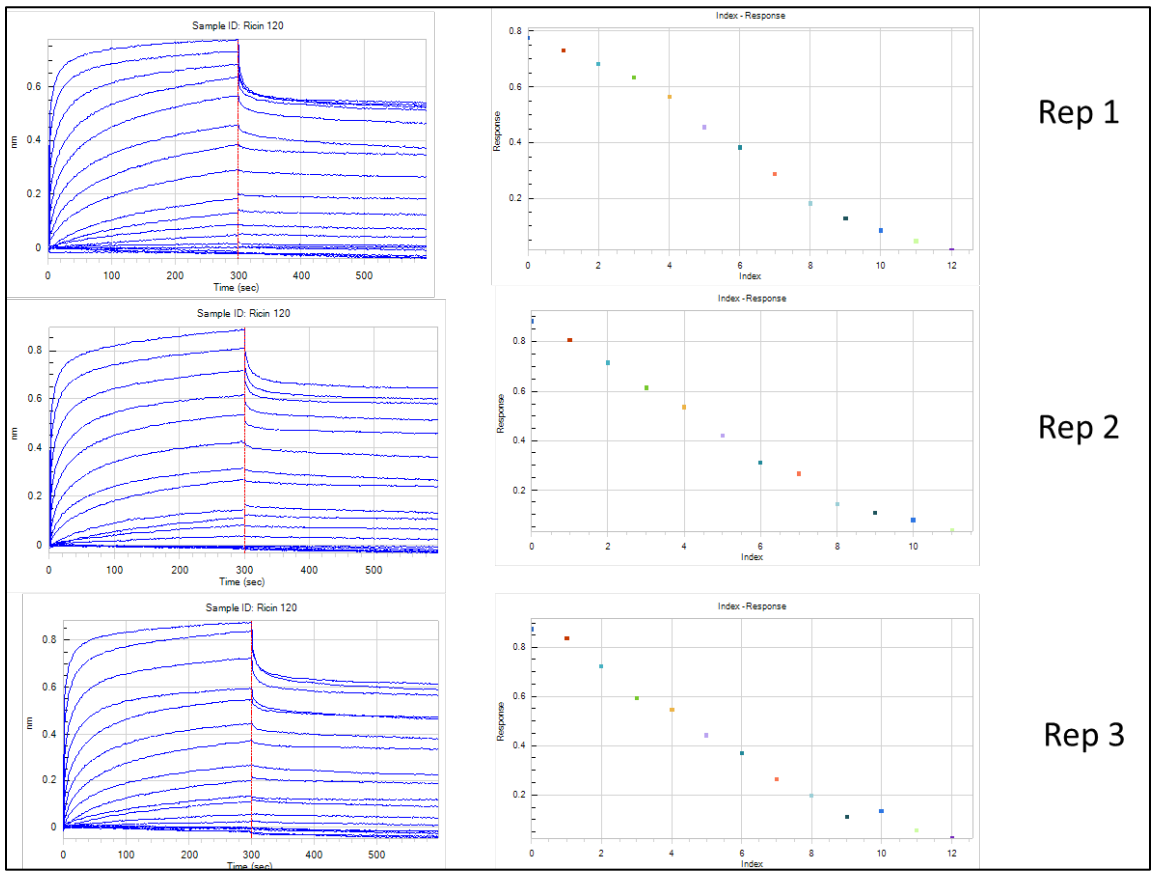

Figure A19. SA sensograms for RCA 120 with anti-ricin Mab 1 in DFU matrix, Rep 1–3.

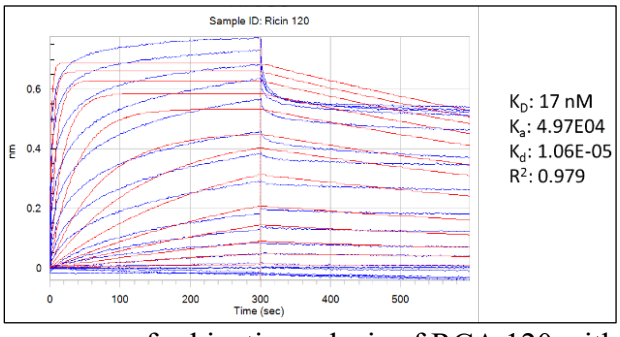

Figure A20. SA sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in DFU matrix.

A.17 AR2G Sensors: RCA 120 in PBS-T

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 238 pg/mL.
- RCA 120 was detected down to 3.81 ng/mL.
- K_D was 3.56 nM.

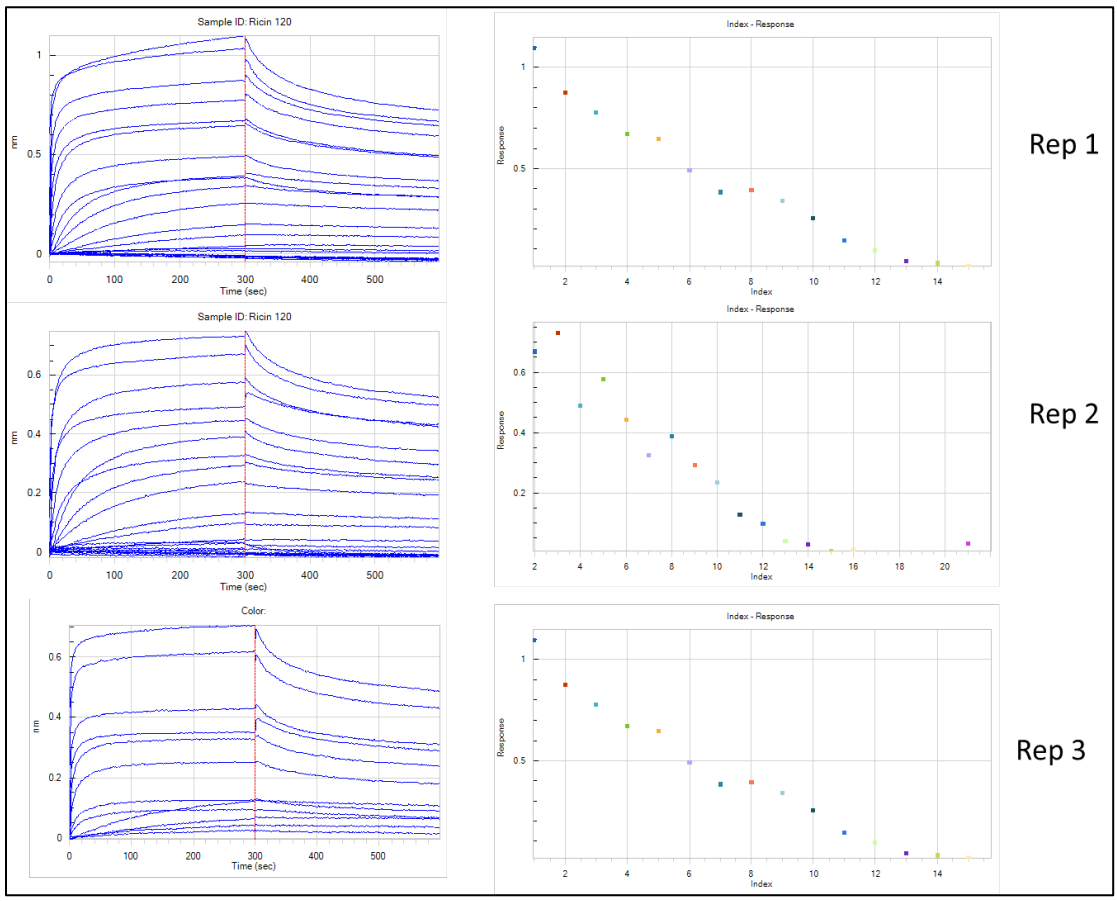

Figure A21. AR2G sensograms for RCA 120 with anti-ricin Mab 1 in PBS-T, Rep 1–3.

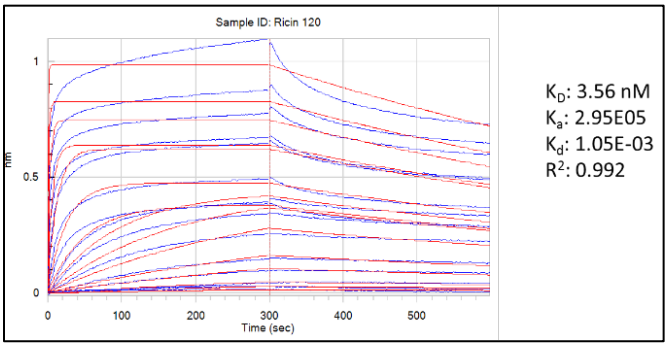

Figure A22. AR2G sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in PBS-T.

A.18 AR2G Sensors: RCA 120 in DFU Matrix

- RCA 120 was diluted in a twofold titration series ranging from 1 mg/mL to 234 pg/mL.
- RCA 120 was detected down to 3.81 ng/mL.
- K_D was 12.4 nM.

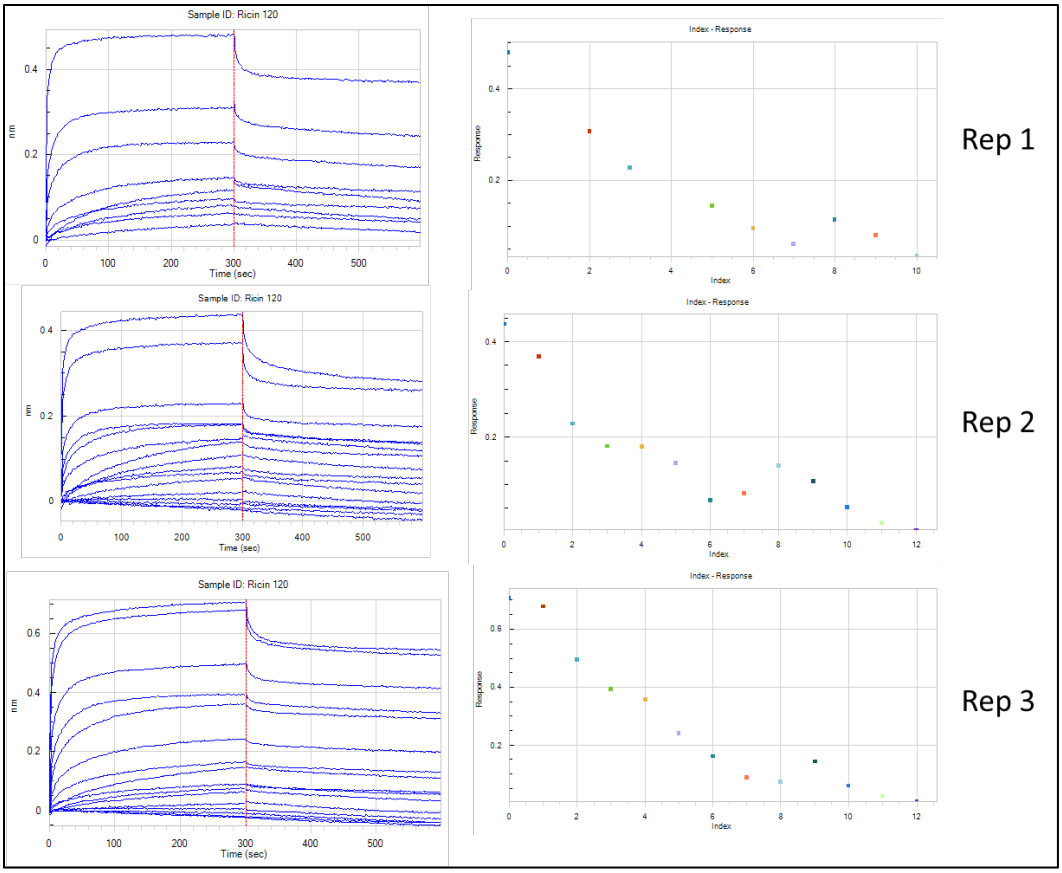

Figure A23. AR2G sensogram for RCA 120 with anti-ricin Mab 1 in DFU matrix, Rep 1–3.

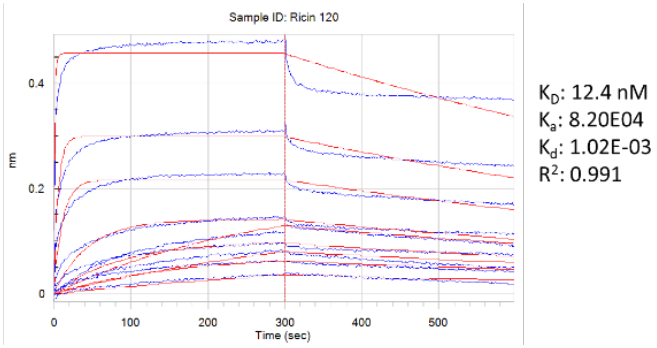

Figure A24. AR2G sensogram for kinetic analysis of RCA 120 with anti-ricin Mab 1 in DFU matrix.

A.19 AMC Sensors: *Bacillus anthracis* in PBS-T

- *B. anthracis* NNR-1 spores were diluted in a twofold titration series ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 3.13E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

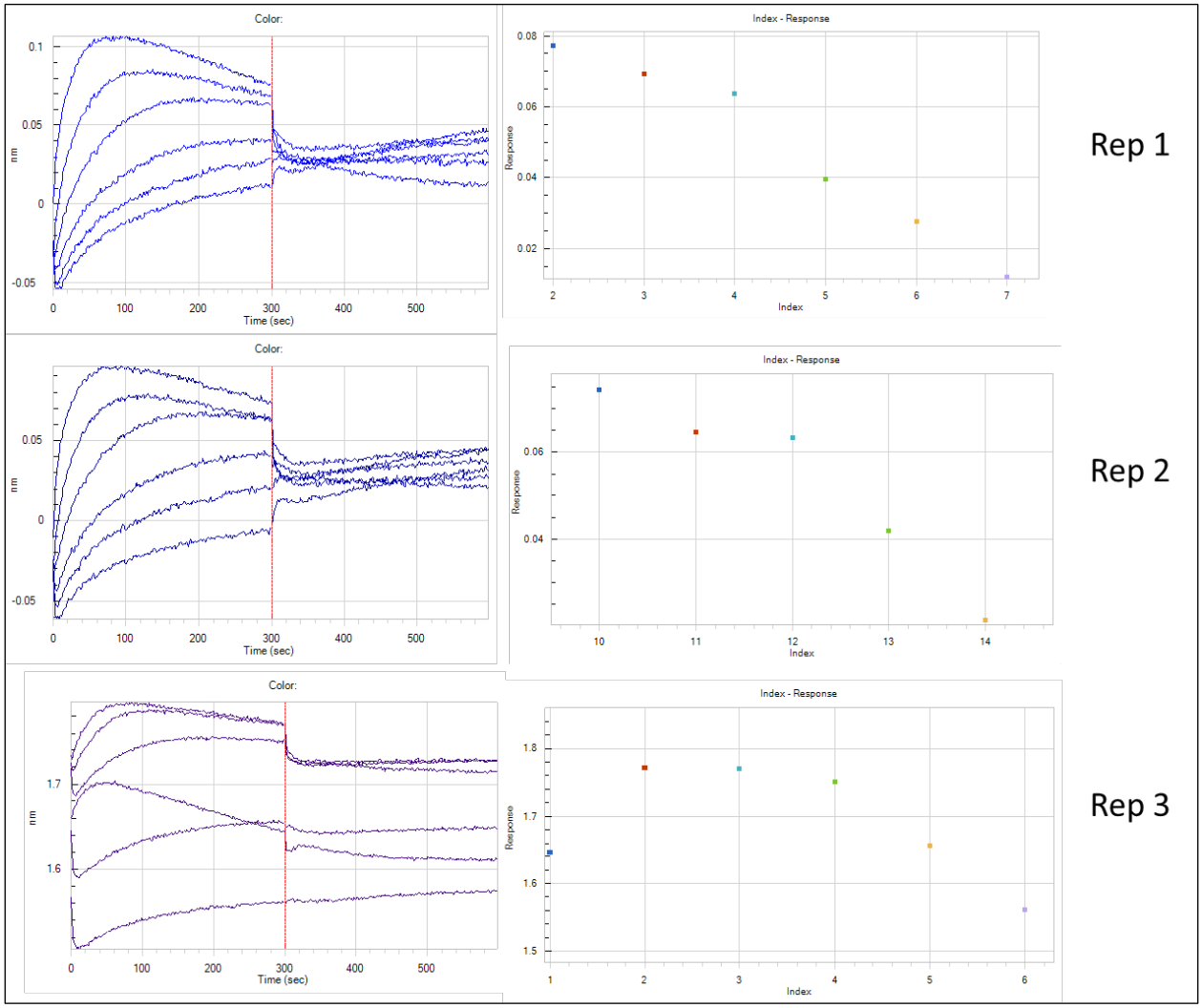

Figure A25. AMC sensogram for *B. anthracis*, NNR-1 spores with anti-*B. anthracis* Mab 4 in PBS-T, Rep 1–3.

A.20 AMC Sensors: *B. anthracis* in DFU Matrix

- *B. anthracis* NNR-1 spores were diluted in a twofold titration ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 5.00E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

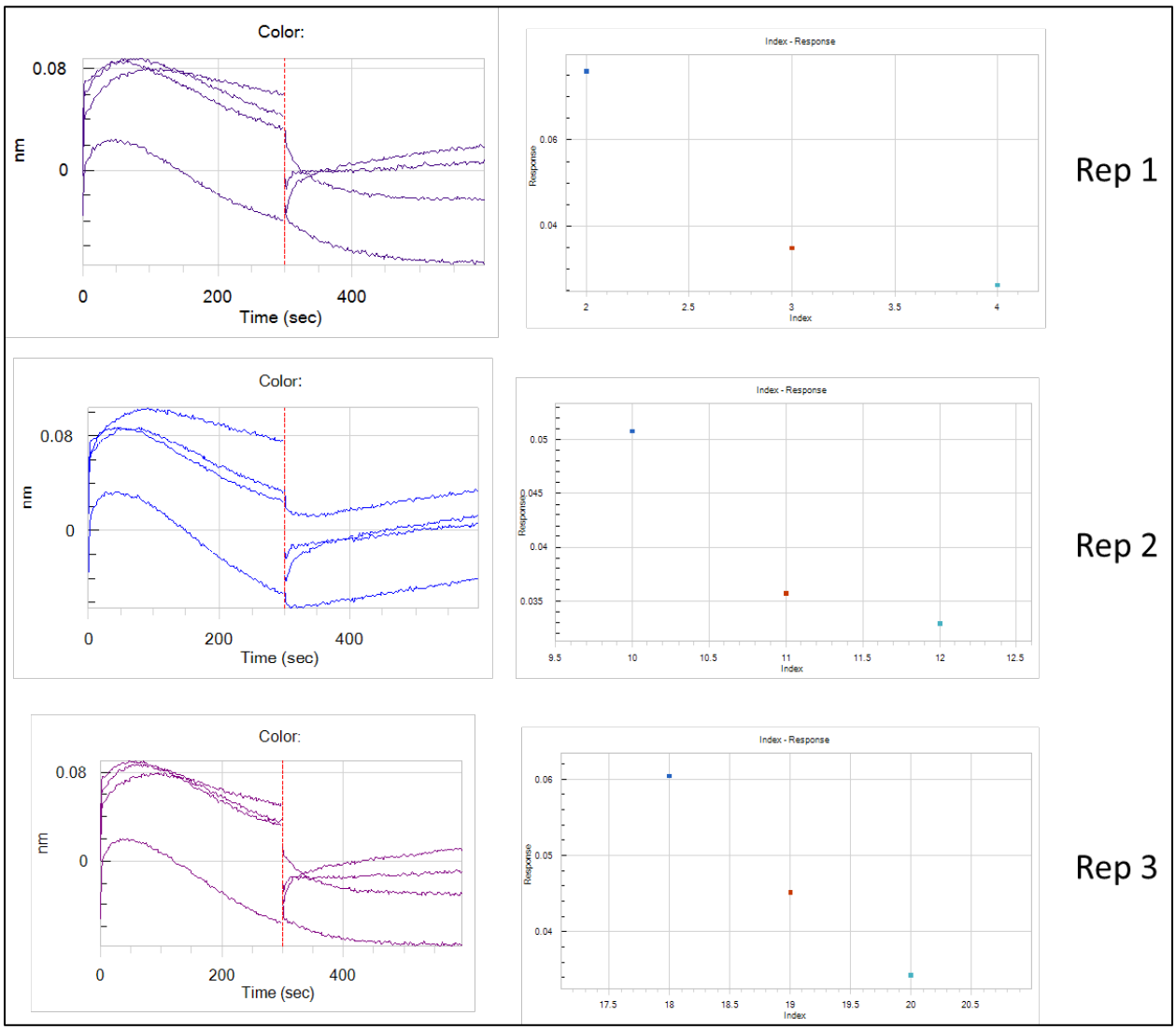

Figure A26. AMC sensogram for *B. anthracis* NNR-1 spores with anti-*B. anthracis* Mab 4 in DFU matrix, Rep 1–3.

A.21 AR2G Sensors: *B. anthracis* in PBS-T

- *B. anthracis* NNR-1 spores were diluted in a twofold titration series ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 6.25E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

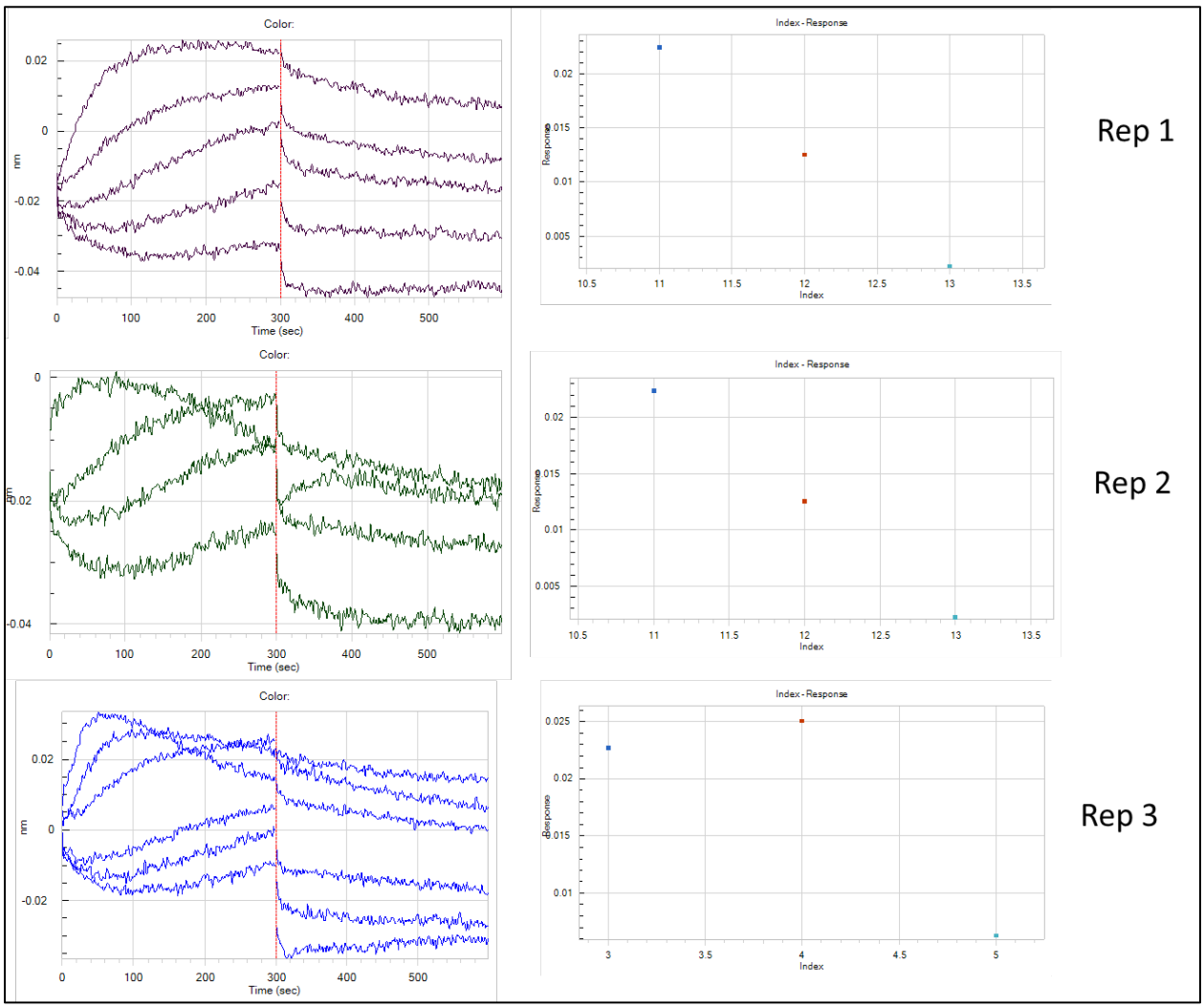

Figure A27. AR2G sensogram for *B. anthracis* NNR-1 spores with anti-*B. anthracis* Mab 4 in PBS-T, Rep 1–3.

A.22 AR2G Sensors: *B. anthracis* in DFU Matrix

- *B. anthracis* NNR-1 spores were diluted in a twofold titration ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 3.13E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

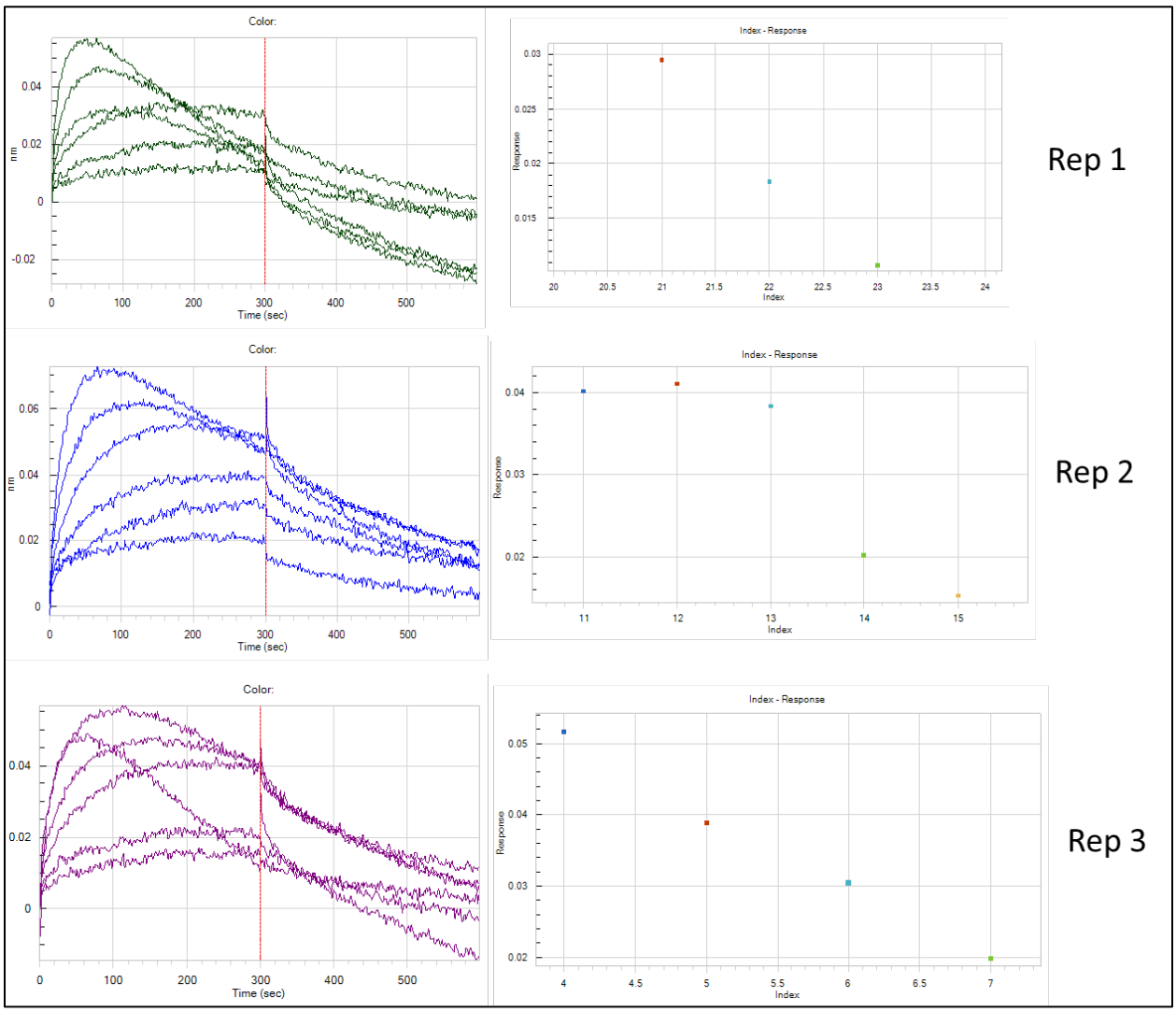

Figure A28. AR2G sensogram for *B. anthracis* NNR-1 spores with anti-*B. anthracis* Mab 4 in DFU matrix, Rep 1–3.

A.23 SA Sensors: *B. anthracis* in PBS-T

- *B. anthracis* NNR-1 spores were diluted in a twofold titration series ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 3.13E+07 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

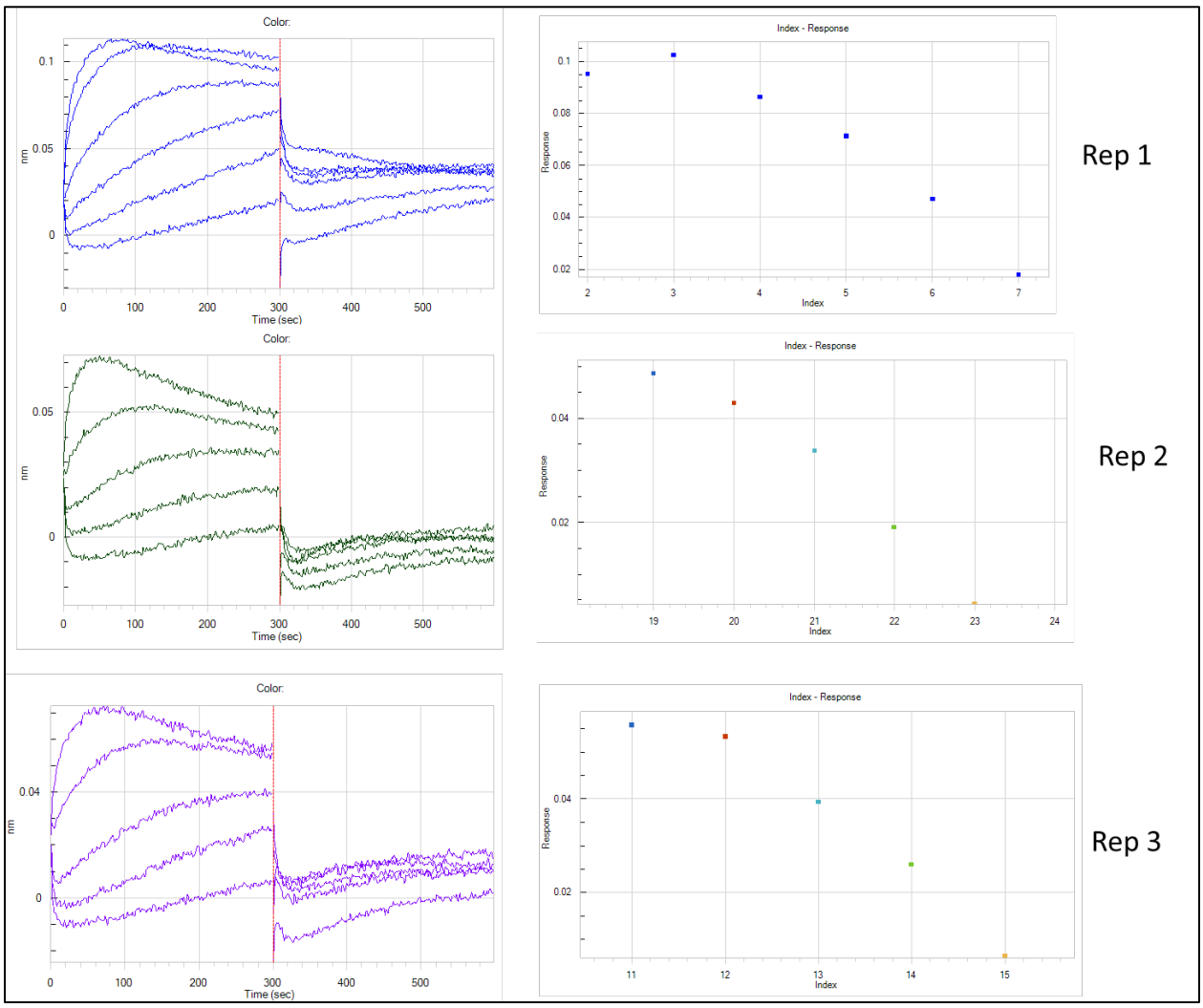

Figure A29. SA sensogram for *B. anthracis* NNR-1 spores with anti-*B. anthracis* Mab 4 in PBS-T, Rep 1–3.

A.24 SA Sensors: *B. anthracis* in DFU Matrix

- *B. anthracis* NNR-1 spores were diluted in a twofold titration series ranging from 2.00E+09 to 1.56E+07 cfu/mL.
- *B. anthracis* was detected down to 2.50E+08 cfu/mL.
- Kinetic analysis was not conducted because whole organisms were used.

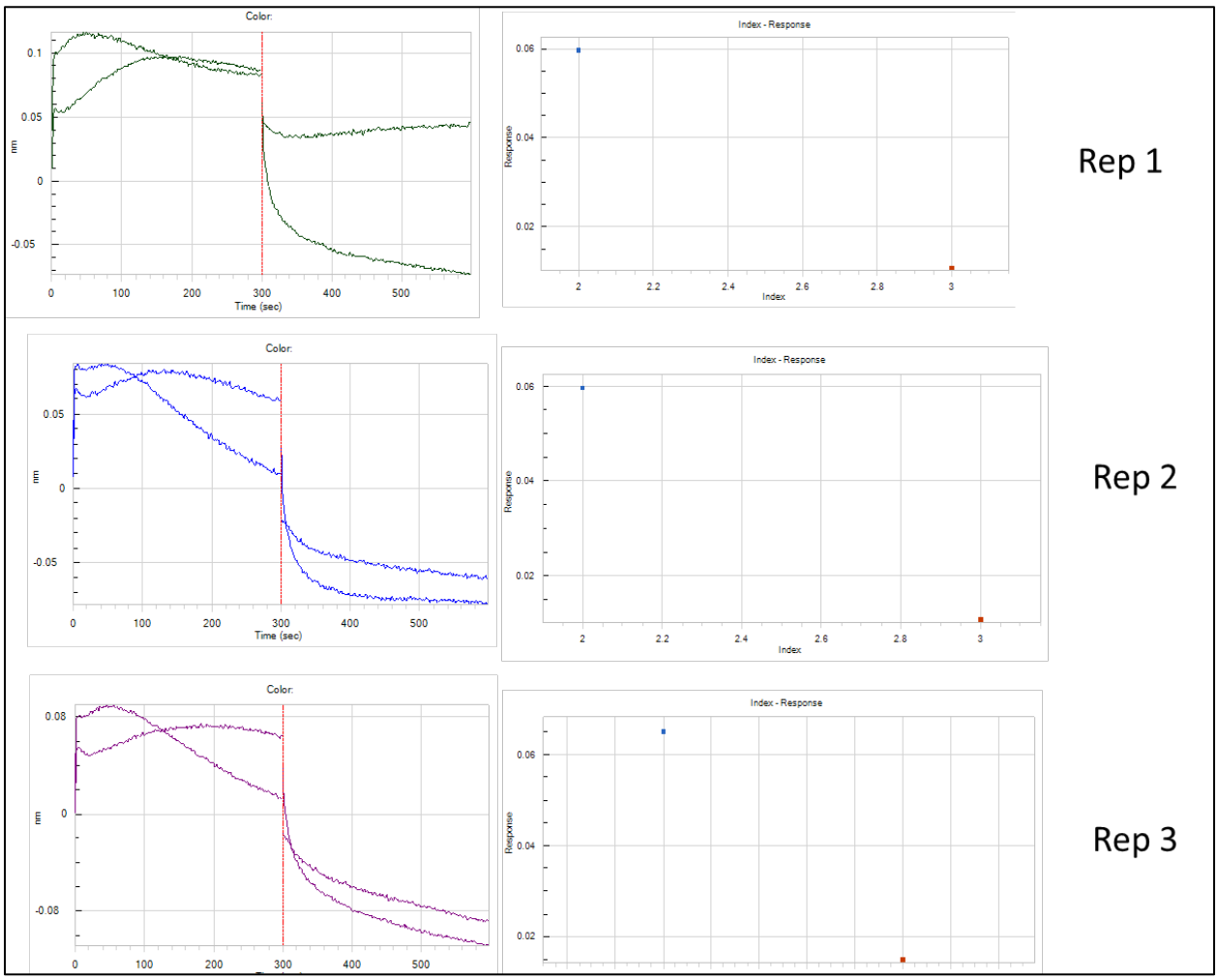

Figure A30. SA sensograms for *B. anthracis* NNR-1 spores with anti-*B. anthracis* Mab 4 in DFU matrix, Rep 1–3.

A.25 AMC Sensors: Ebola Virus-Like Particles (VLPs) in PBS-T

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL.
- Ebola VLPs were detected down to 31.25 µg/mL.
- K_D was 255 nM.

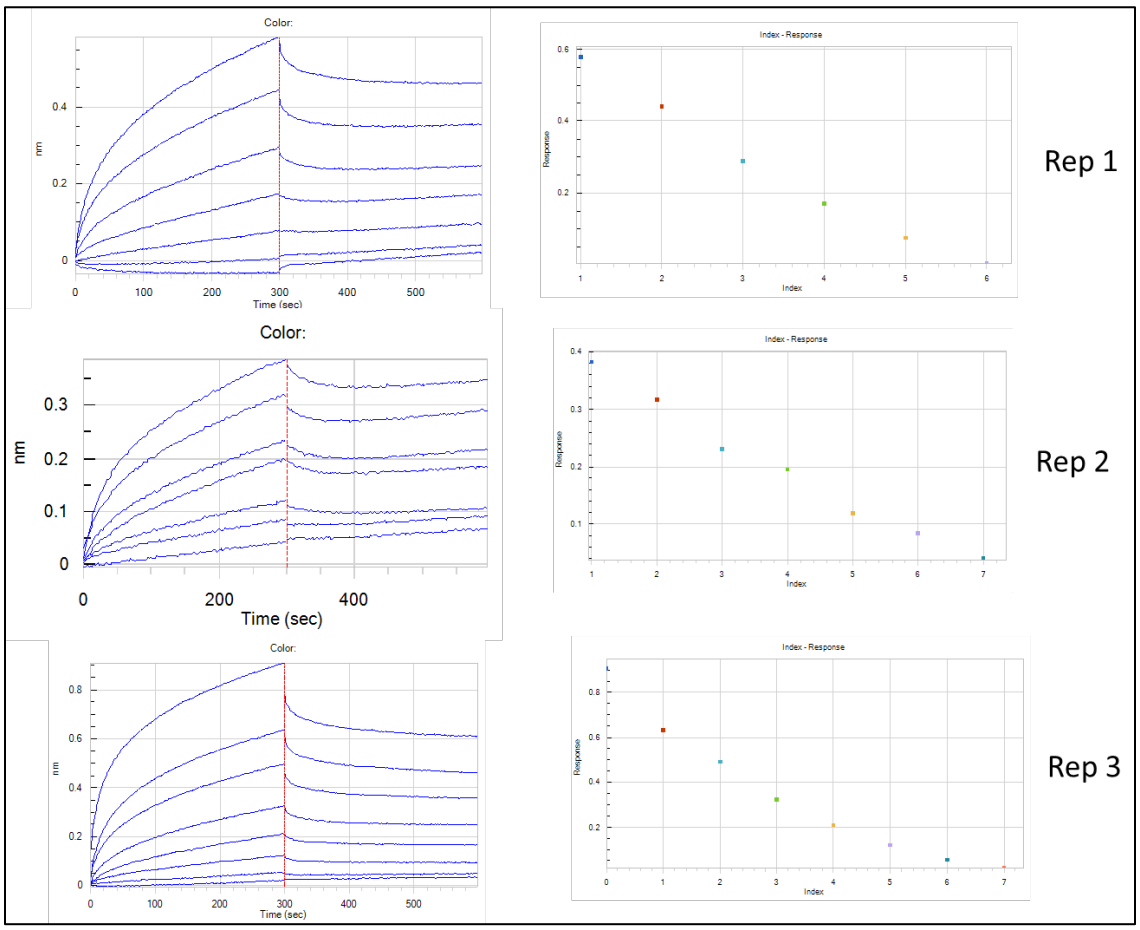

Figure A31. AMC sensogram for Ebola VLPs with anti-Ebola Mab 2 in PBS-T, Rep 1–3.

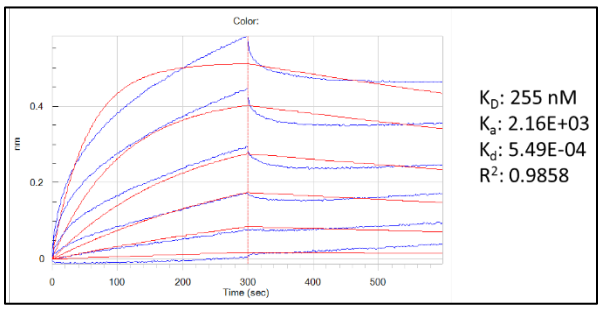

Figure A32. AMC sensor kinetic analysis of Ebola VLPs with anti-Ebola Mab2 in PBS-T.

A.26 AMC Sensors: Ebola VLPs in DFU Matrix

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL
- Ebola VLPs were detected down to 7.81 µg/mL.
- K_D was 1 nM.

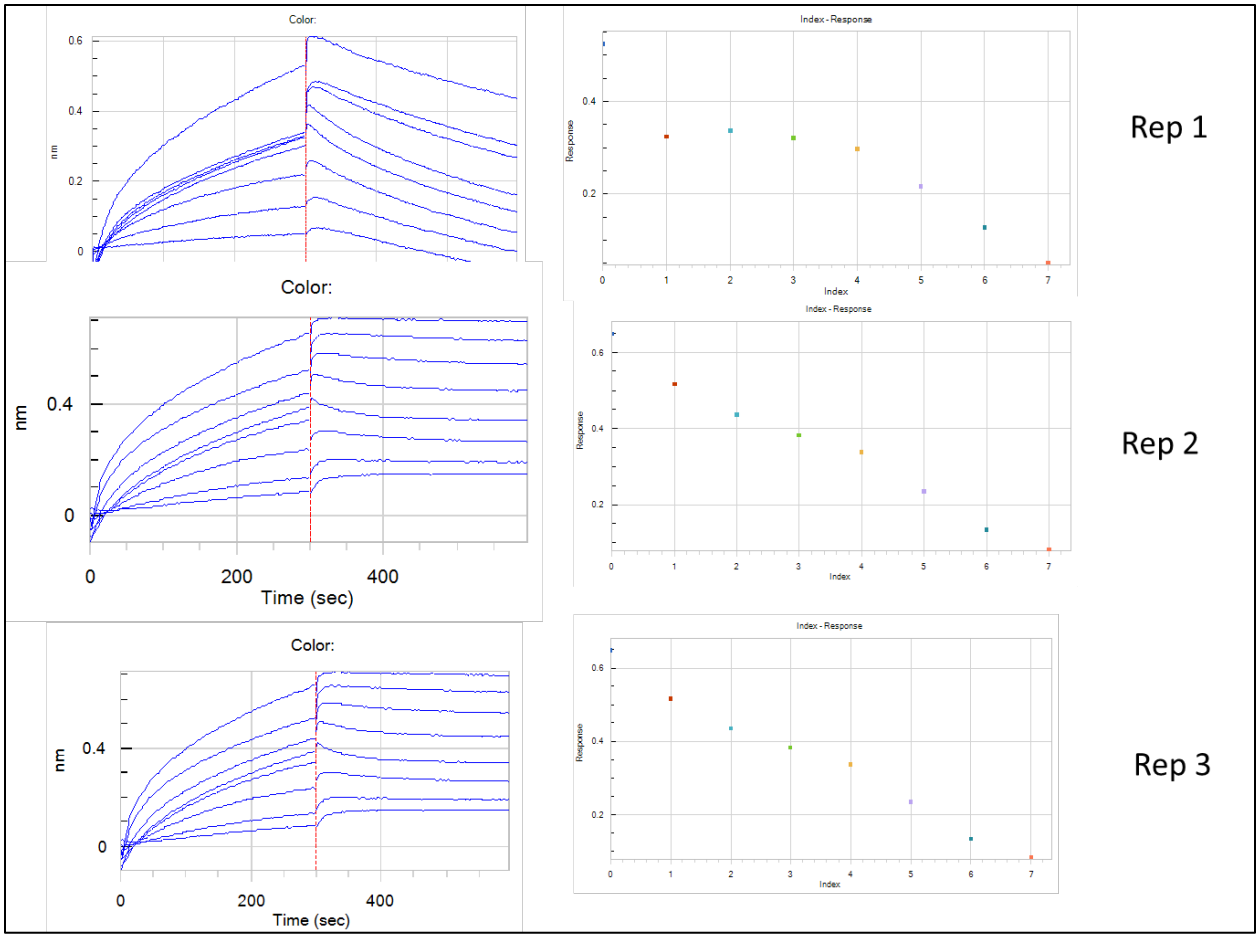

Figure A33. AMC sensogram for Ebola VLPs with anti-Ebola Mab 2 in DFU matrix, Rep 1–3.

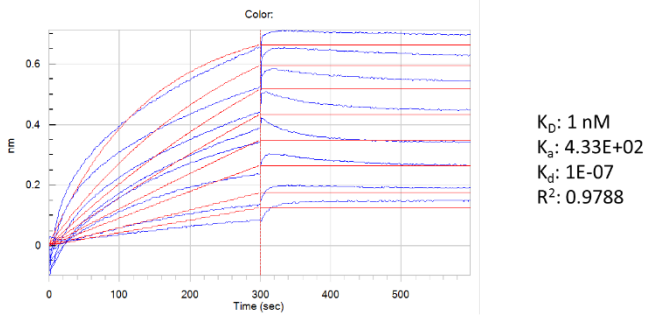

Figure A34. AMC sensor kinetic analysis of Ebola VLPs with anti-Ebola Mab 2 in DFU matrix.

A.27 SA Sensors: Ebola VLPs in PBS-T

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL
- Ebola VLPs were detected down to 31.25 µg/mL.
- K_D was 813 nM.

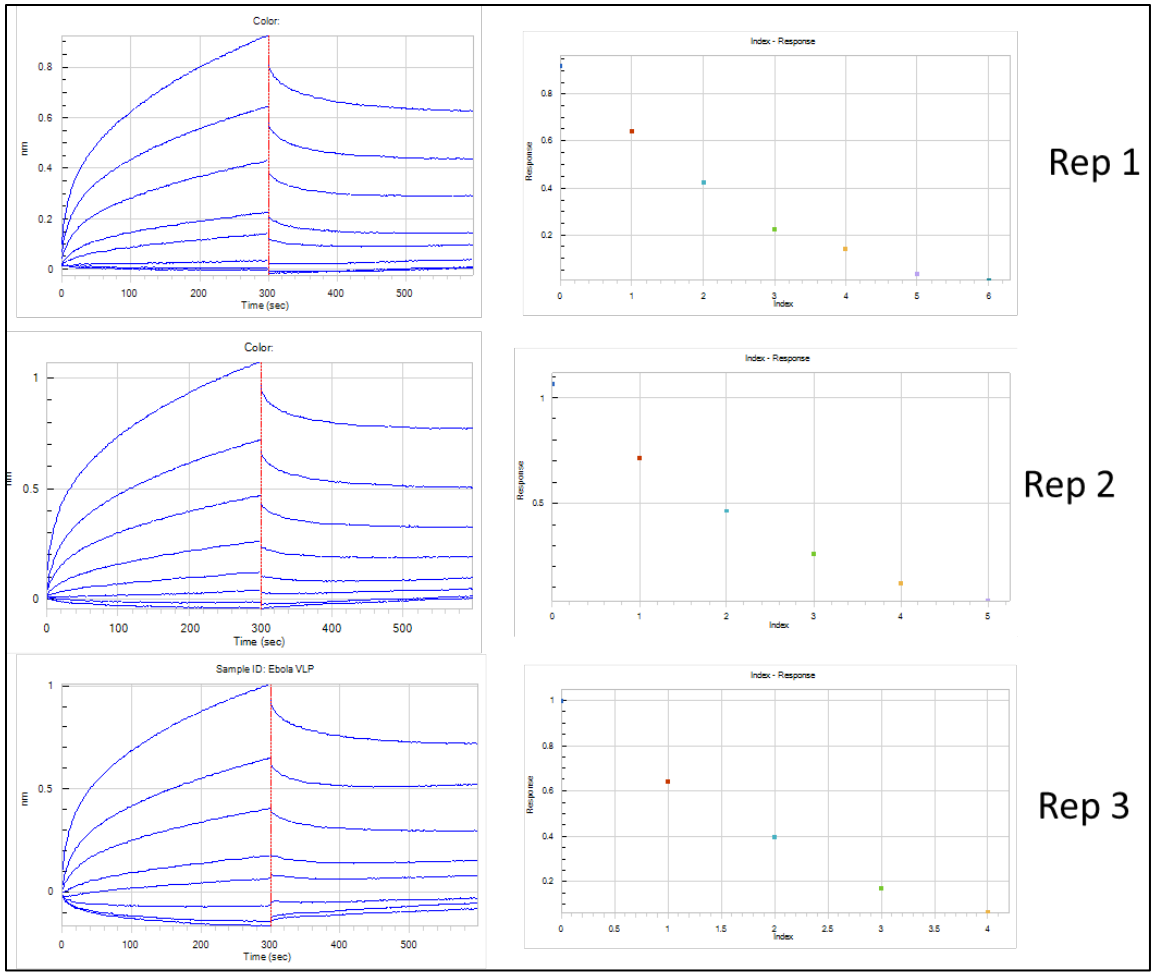

Figure A35. SA sensogram for Ebola VLPs with anti-Ebola Mab 2 in PBS-T, Rep 1–3.

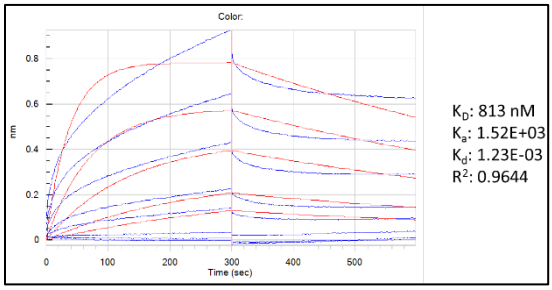

Figure A36. SA sensors kinetic analysis of Ebola VLPs with anti-Ebola Mab 2 in PBS-T.

A.28 SA Sensors: Ebola VLPs in DFU Matrix

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL.
- Ebola VLPs were detected down to 15.63 µg/mL.
- K_D was 1 nM.

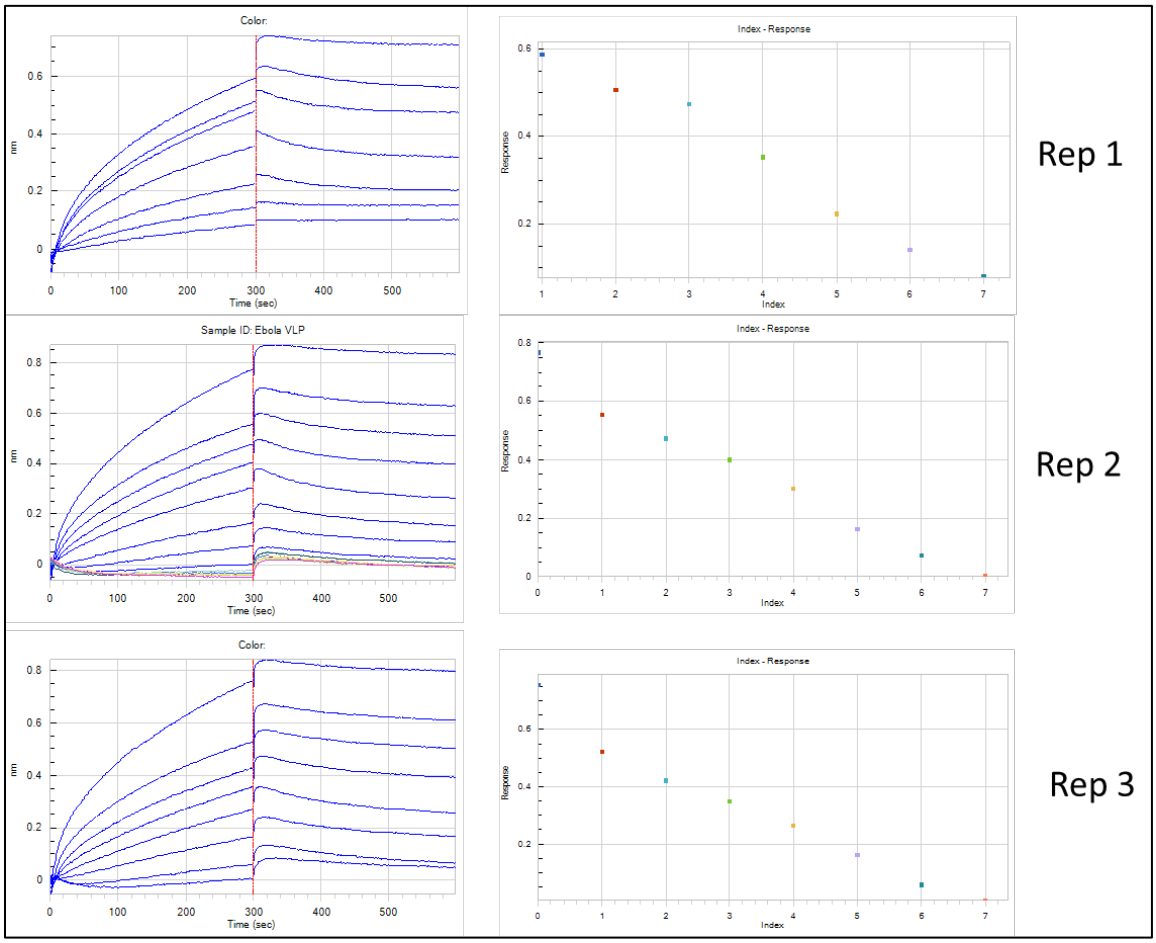

Figure A37. SA sensogram for Ebola VLPs with anti-Ebola Mab 2 in DFU matrix, Rep 1–3.

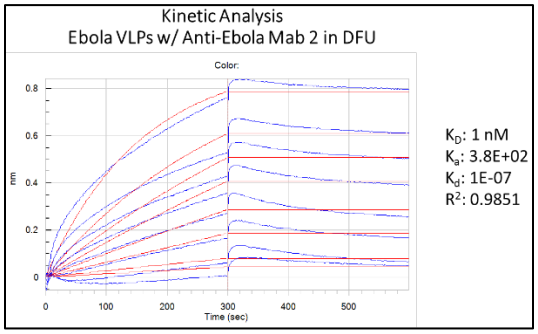

Figure A38. SA sensor kinetic analysis of Ebola VLPs with anti-Ebola Mab 2 in DFU matrix.

A.29 AR2G Sensors: Ebola VLPs in PBS-T

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL.
- Ebola VLPs were detected down to 31.25 µg/mL.
- K_D was 481 nM.

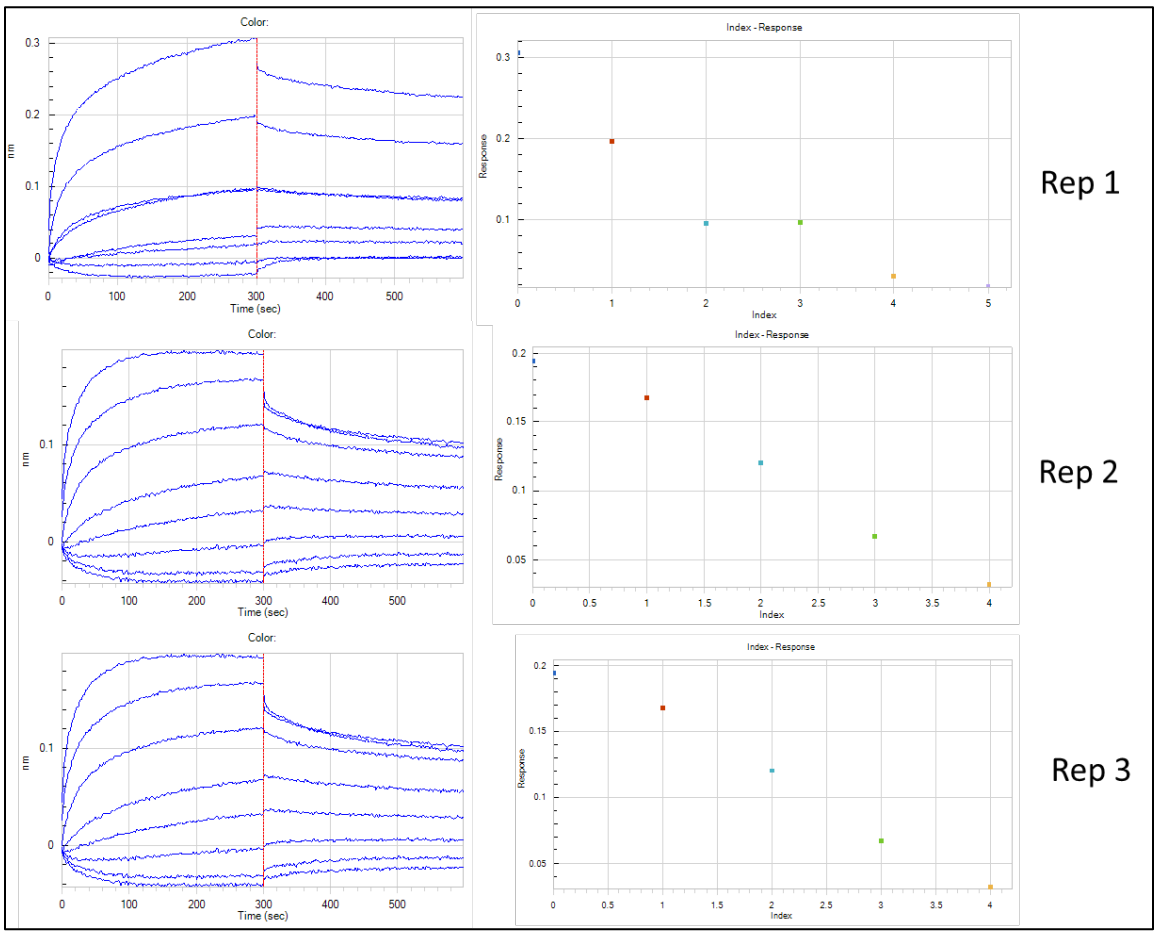

Figure A39. AR2G sensogram for Ebola VLPs with anti-Ebola Mab 2 in PBS-T, Rep 1–3.

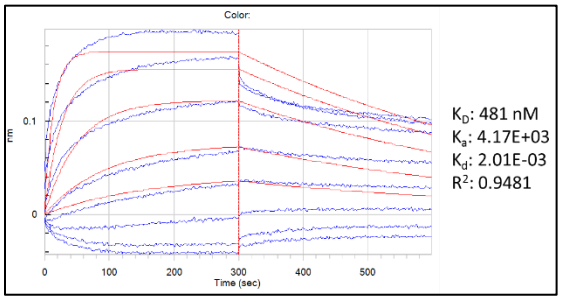

Figure A40. AR2G Sensor kinetic analysis Ebola VLPs with anti-Ebola Mab 2.

A.30 AR2G Sensors: Ebola VLPs in DFU Matrix

- Ebola VLPs were diluted in a twofold titration series ranging from 1 mg/mL to 61 ng/mL
- Ebola VLPs were detected down to 62.5 µg/mL
- K_D was 1.25 nM

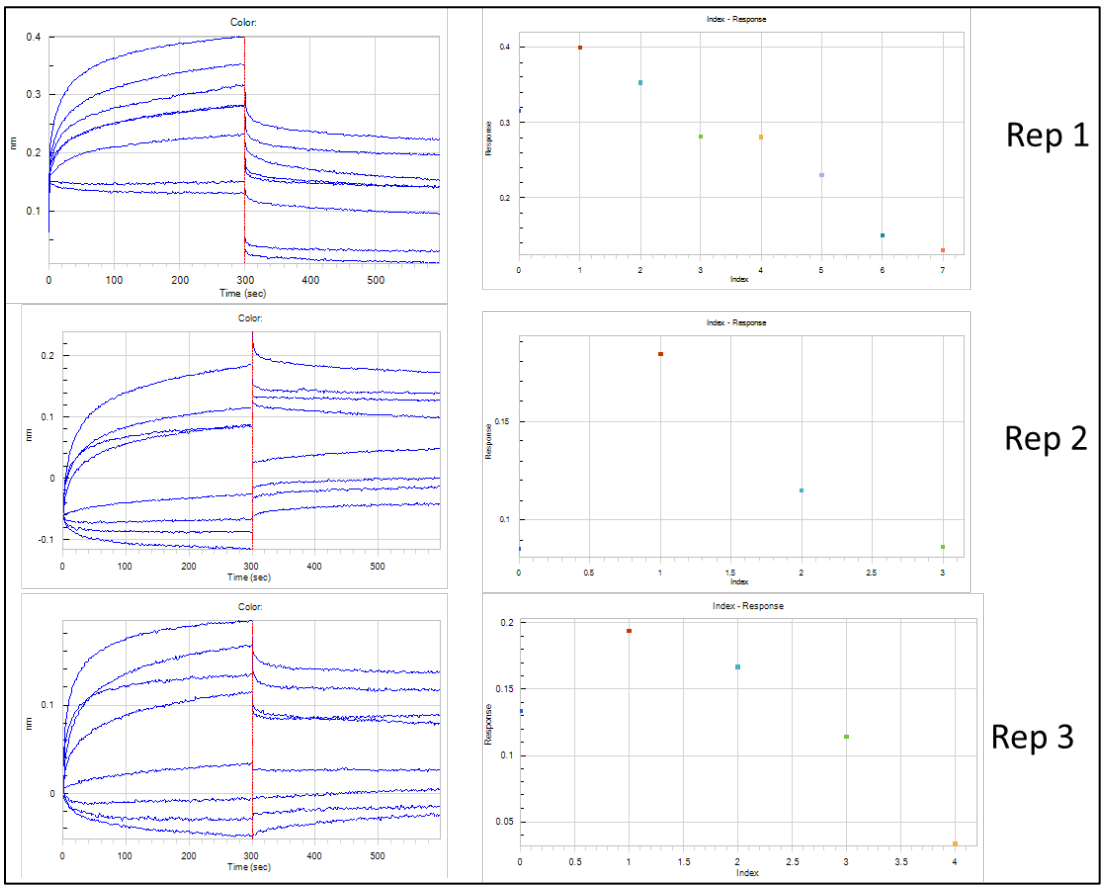

Figure A41. AR2G sensogram for Ebola VLPs with anti-Ebola Mab 2 DFU matrix, Rep 1–3.

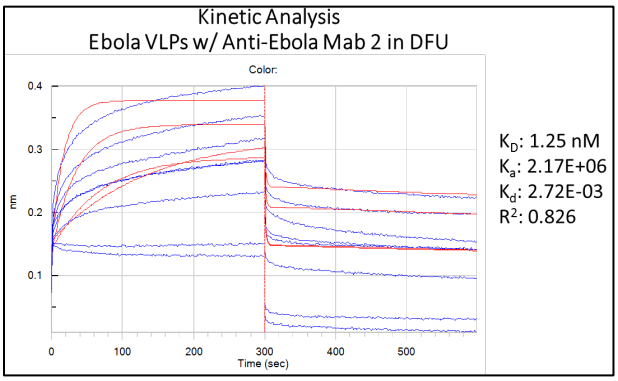

Figure A42. AR2G sensor kinetic analysis Ebola VLPs with anti-Ebola Mab 2 in DFU matrix.

A.31 AMC Sensor: T2 Mycotoxin in PBS-T

- T2 mycotoxin was diluted in a twofold titration series that started with 1 mg/mL and ended in 7.8 µg/mL.
- T2 mycotoxin was detected down to 31.25 µg/mL.
- Limit of detection (LOD) determination was made using the response versus sensor (index) graph as well as the affinity graph.

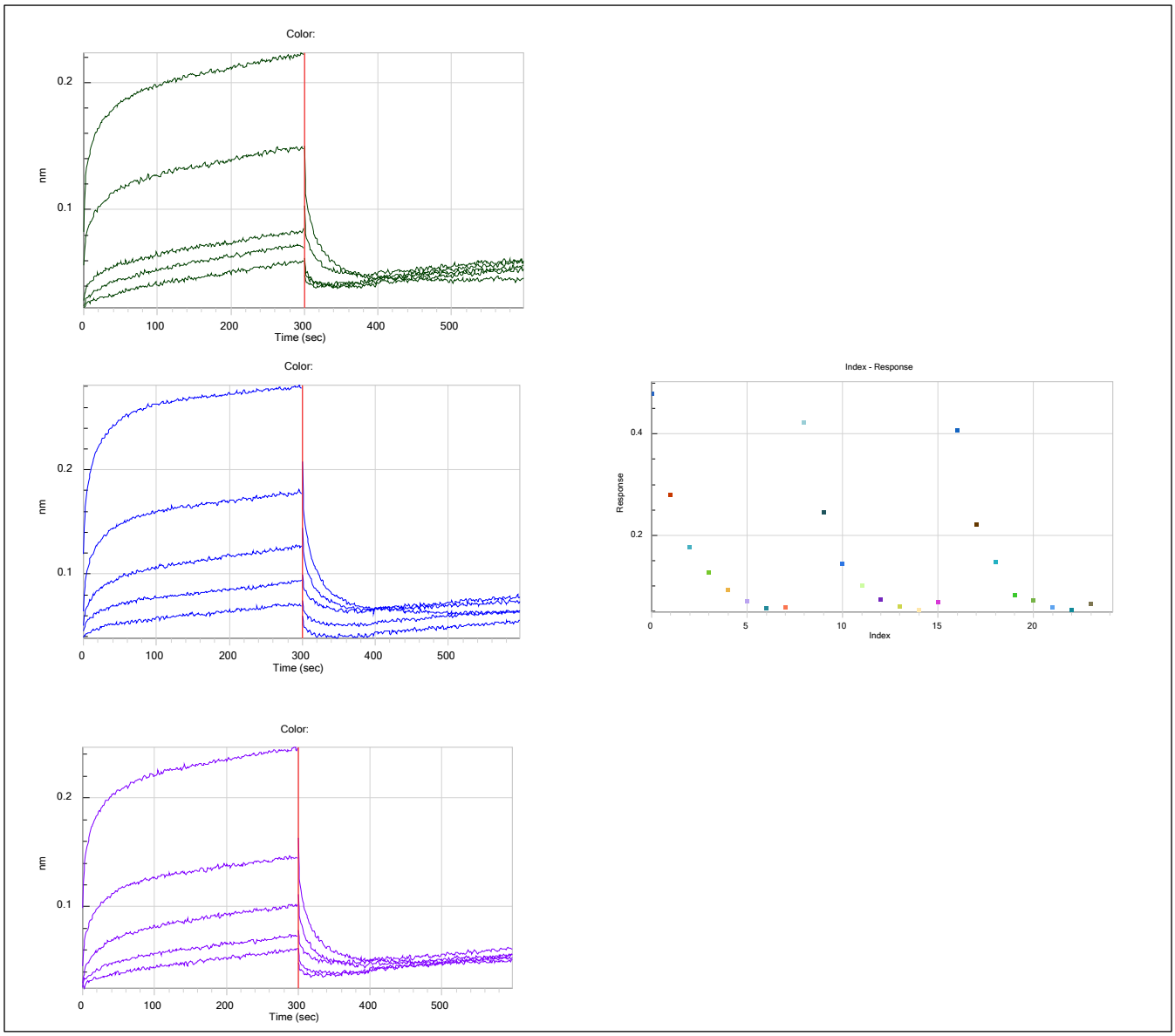

Figure A43. AMC sensogram for T2 mycotoxin with anti-T2 Mab 1 in PBS-T, Rep 1–3.

A.32 AMC Sensors: T2 Mycotoxin in DFU Matrix

- T2 mycotoxin was diluted in a twofold titration series ranging from 500 to 15.63 µg/mL.
- T2 mycotoxin was detected down to 500 µg/mL.
- LOD determination was made using the response versus sensor (index) graph as well as the affinity graph.

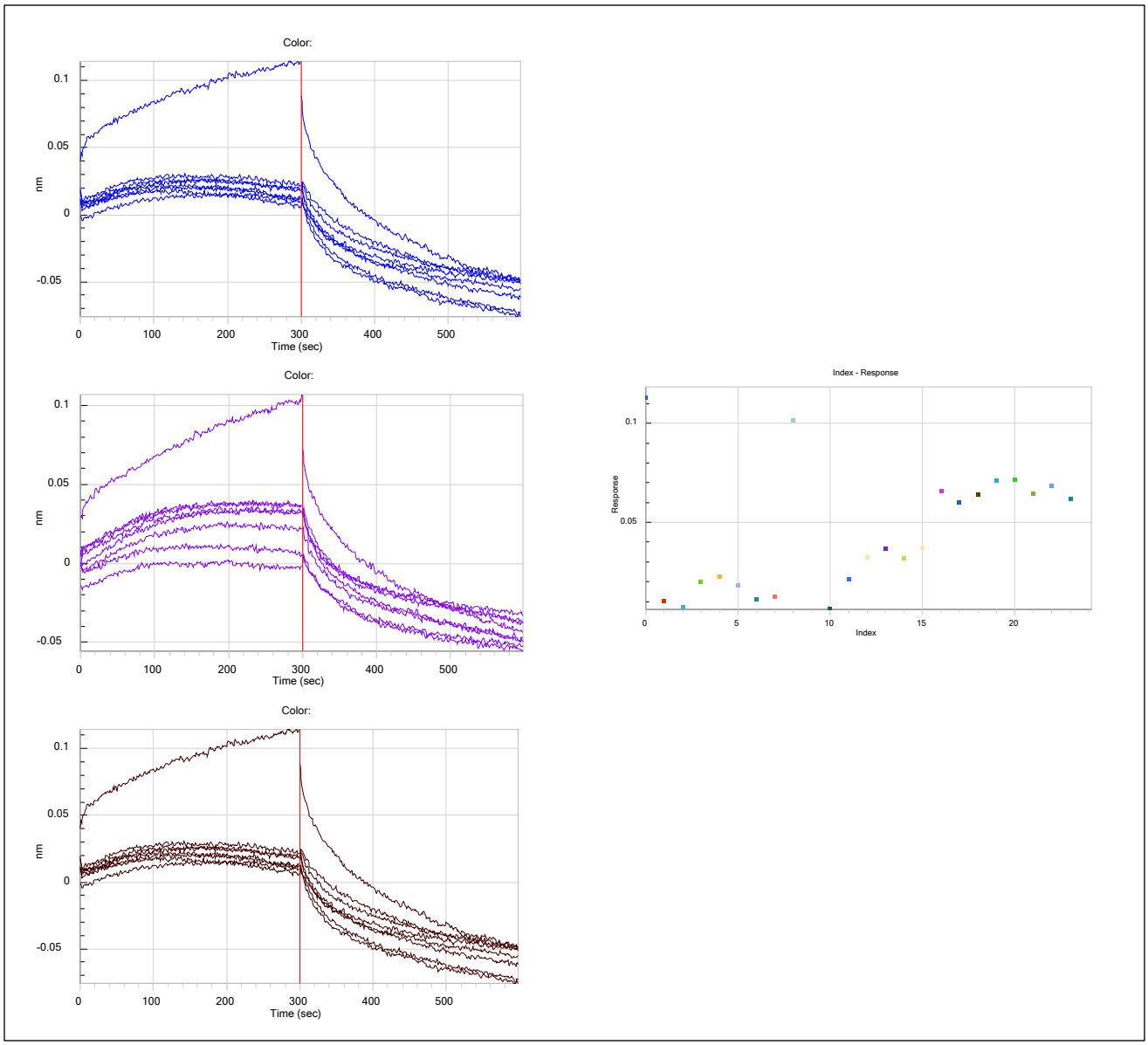

Figure A44. AMC sensogram for T2 mycotoxin with anti-T2 Mab 1 in DFU matrix, Rep 1–3.

A.33 SA Sensors: T2 Mycotoxin in PBS-T

- T2 mycotoxin was diluted in a twofold titration series ranging from 1 mg/mL to 7.8 µg/mL.
- T2 mycotoxin was detected down to 62.5 µg/mL.
- LOD determination was made using the response versus sensor (index) graph as well as the affinity graph.

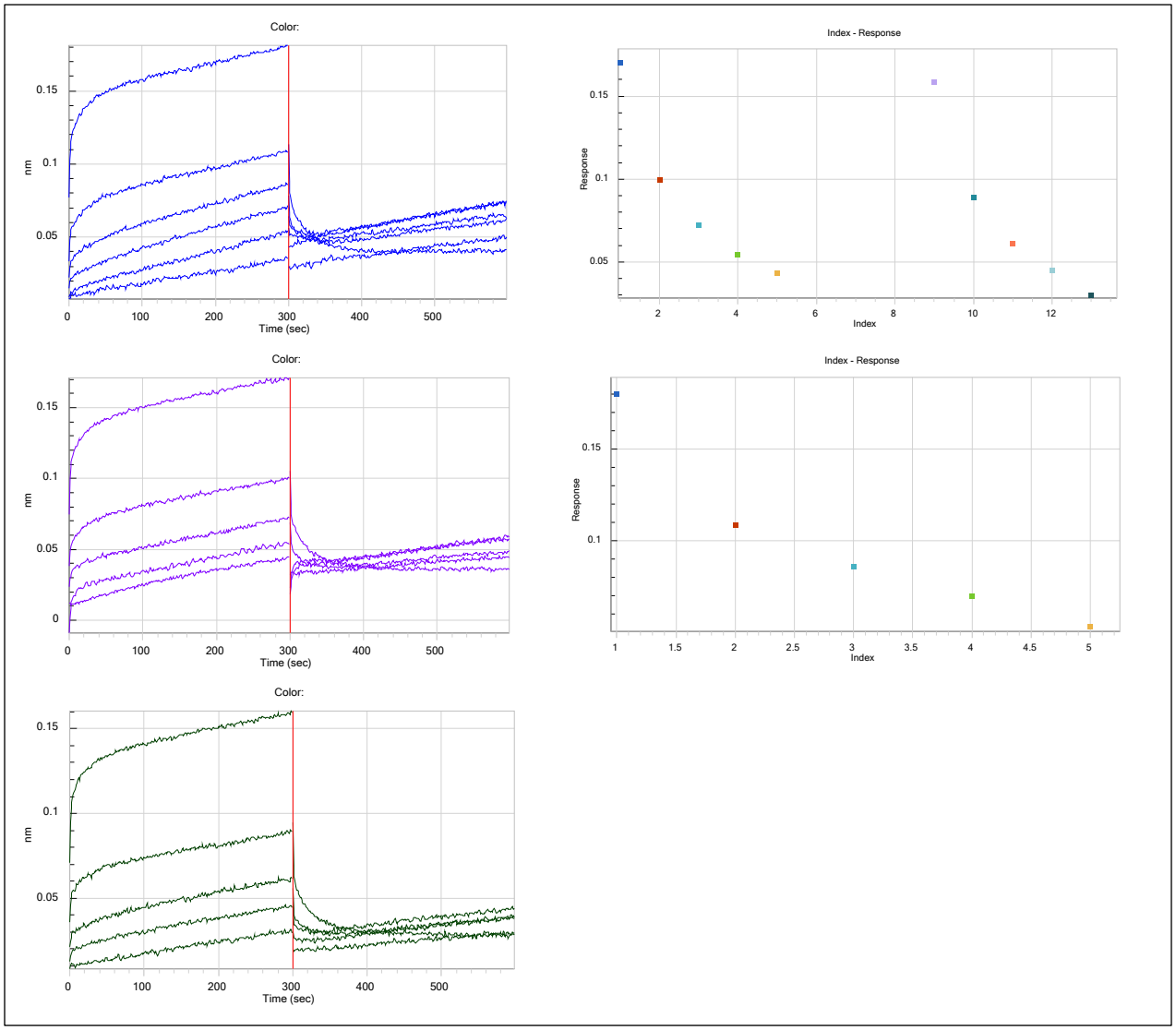

Figure A45. SA sensogram for T2 mycotoxin with anti-T2 Mab 1 in PBS-T, Rep 1–3.

A.34 SA Sensors: T2 Mycotoxin in DFU Matrix

- T2 mycotoxin was diluted in a twofold titration series ranging from 1 mg/mL to 7.8 µg/mL.
- T2 mycotoxin was detected down to 62.5 µg/mL.
- LOD determination was made using the Response versus sensor (index) graph as well as the affinity graph.

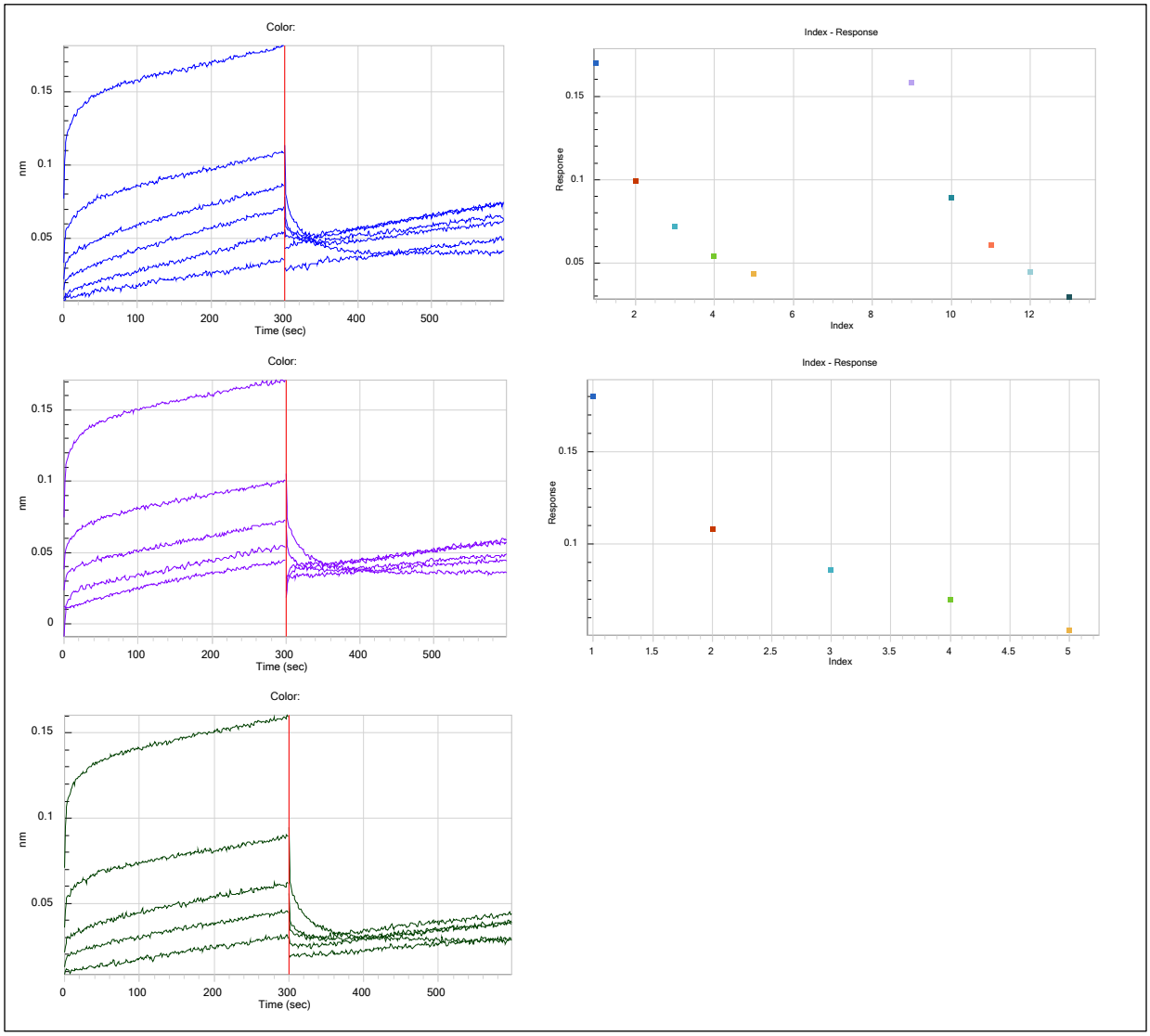

Figure A46. SA sensogram for T2 mycotoxin with anti-T2 Mab 1 in PBS-T, Rep 1–3.

A.35 SA Sensors: T2 Mycotoxin in DFU Matrix

- T2 mycotoxin was diluted in a twofold titration series ranging from 500 to 15.63 µg/mL.
- T2 mycotoxin was detected down to 500 µg/mL.
- LOD determination was made using the response versus sensor (index) graph as well as the affinity graph.

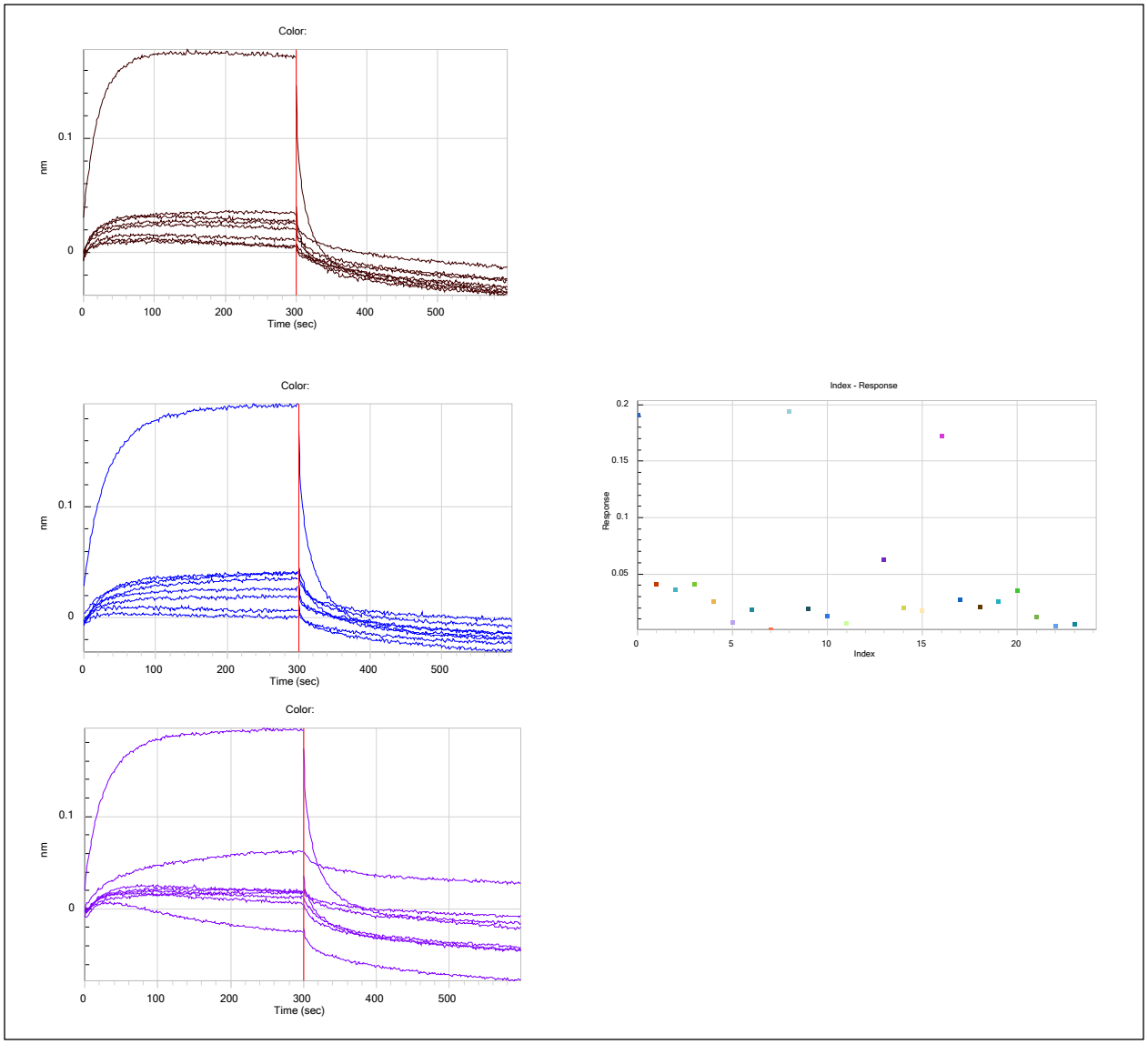

Figure A47. SA sensogram for T2 mycotoxin with anti-Mab 1 in DFU matrix, Rep 1–3.

A.36 AR2G Sensors: T2 Mycotoxin in PBS-T

- T2 mycotoxin was diluted in a twofold titration series ranging from 1 mg/mL to 7.8 µg/mL.
- T2 mycotoxin was detected down to 500 µg/mL.
- LOD determination was made using the response versus sensor (index) graph as well as the affinity graph.

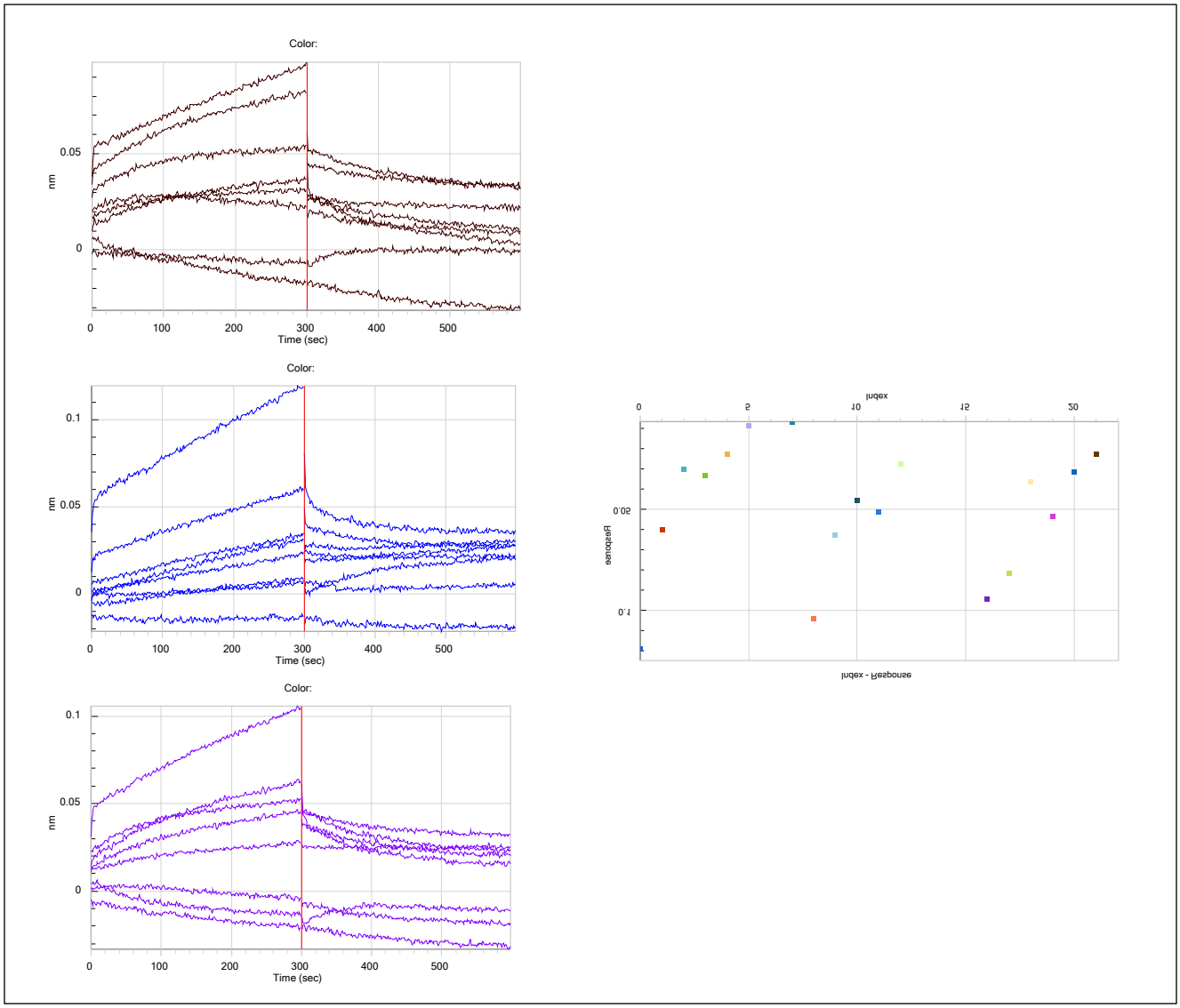

Figure A48. AR2G sensogram for T2 mycotoxin with anti-T2 Mab 1 in PBS-T, Rep 1–3.

A.37 AR2G Sensors: T2 Mycotoxin in DFU Matrix

- T2 mycotoxin was diluted in a twofold titration series ranging from 500 to 15.63 µg/mL.
- T2 mycotoxin was detected down to 500 µg/mL.
- LOD determination was made using the response vs sensor (index) graph as well as the affinity graph.

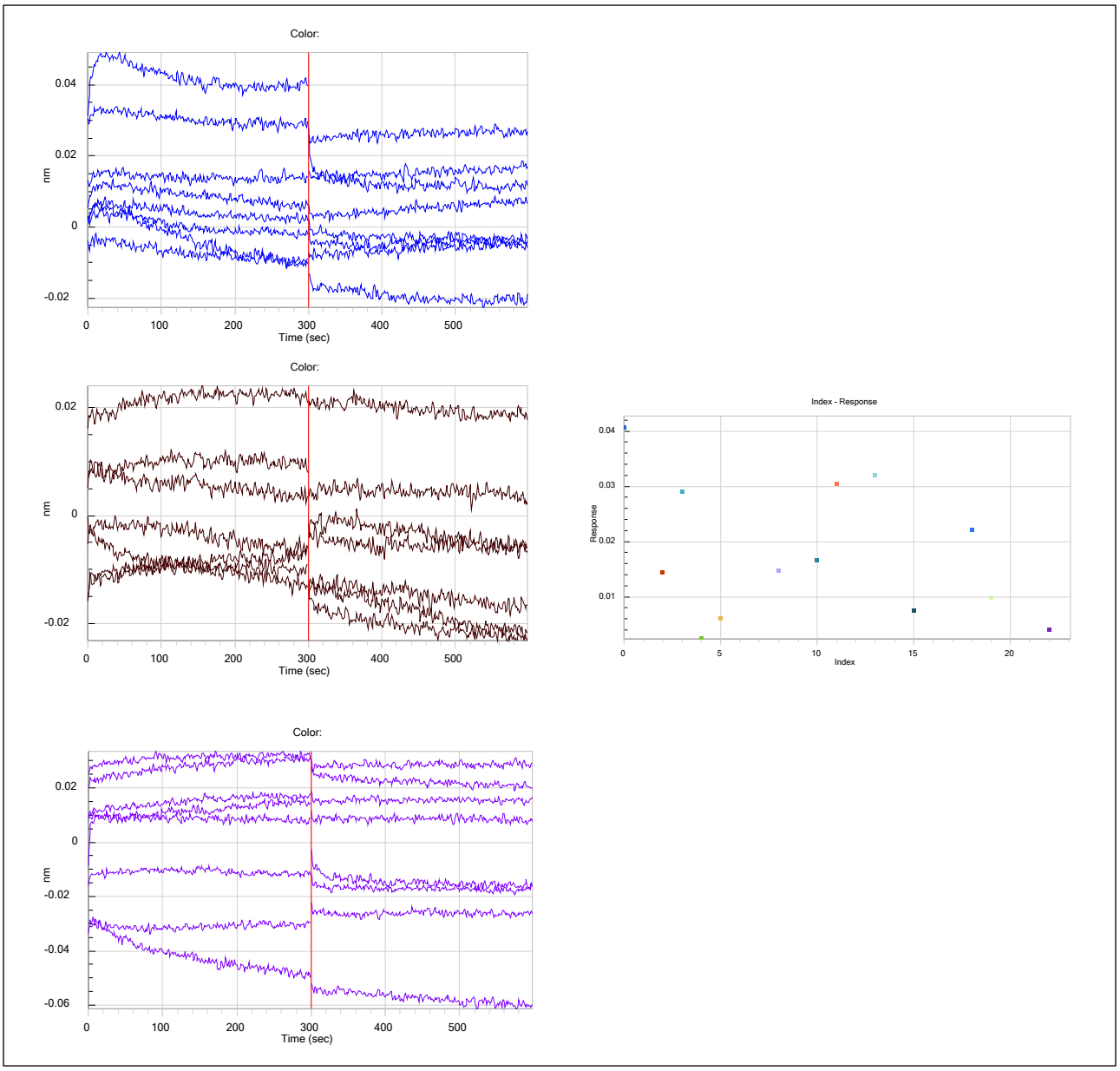

Figure A49. AR2G sensogram for T2 mycotoxin with anti-T2 Mab 1 in DFU matrix, Rep 1–3.

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APPENDIX B

COCKTAIL GRAPHS

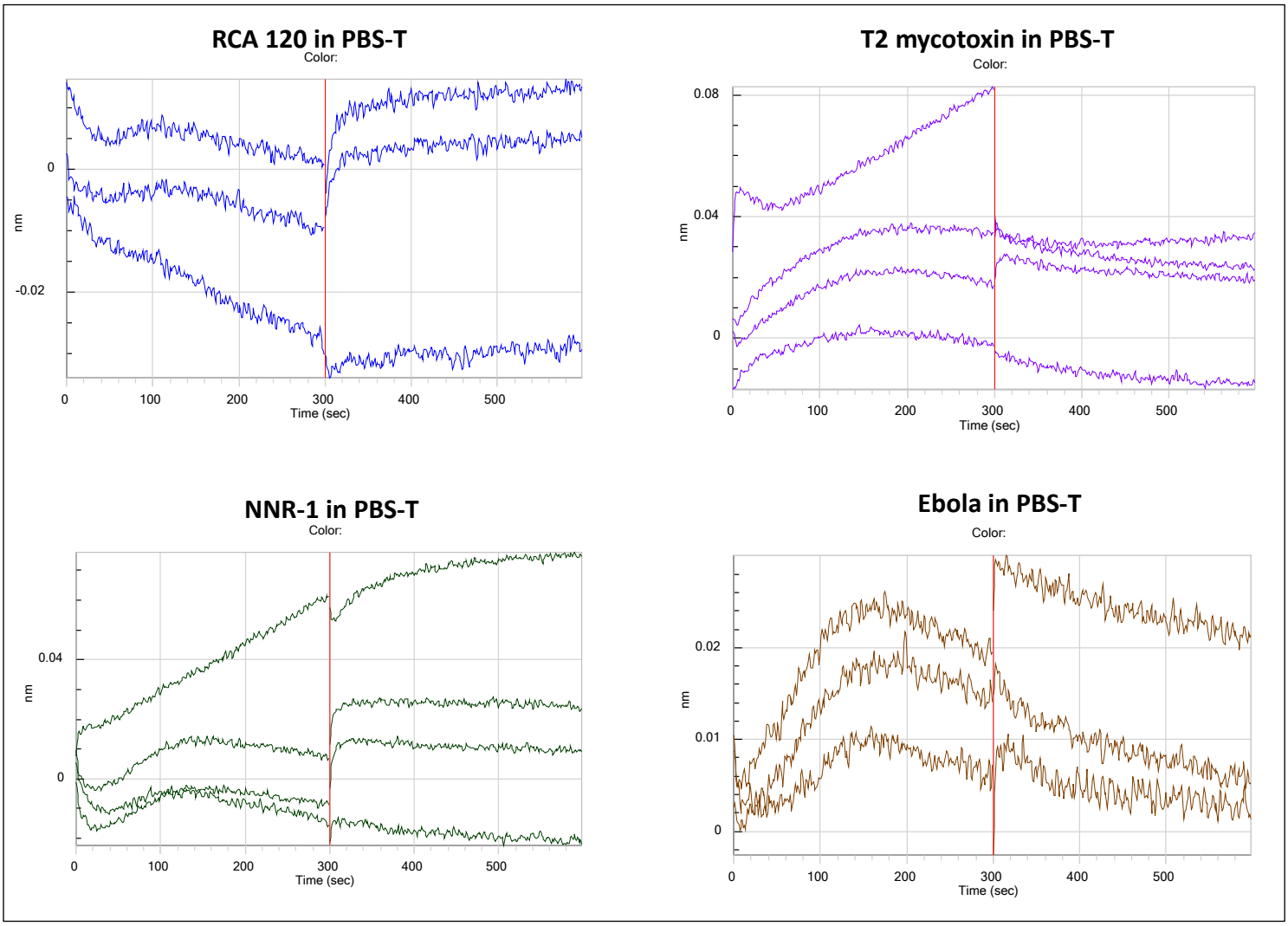

Figure B1. Cocktail 1: *Ricinus communis* agglutinin (RCA 120), T2 mycotoxin, *Bacillus anthracis* NNR-1, and Ebola virus-like particles (VLPs) in phosphate-buffered saline with 0.1% Triton X-100 (PBS-T).

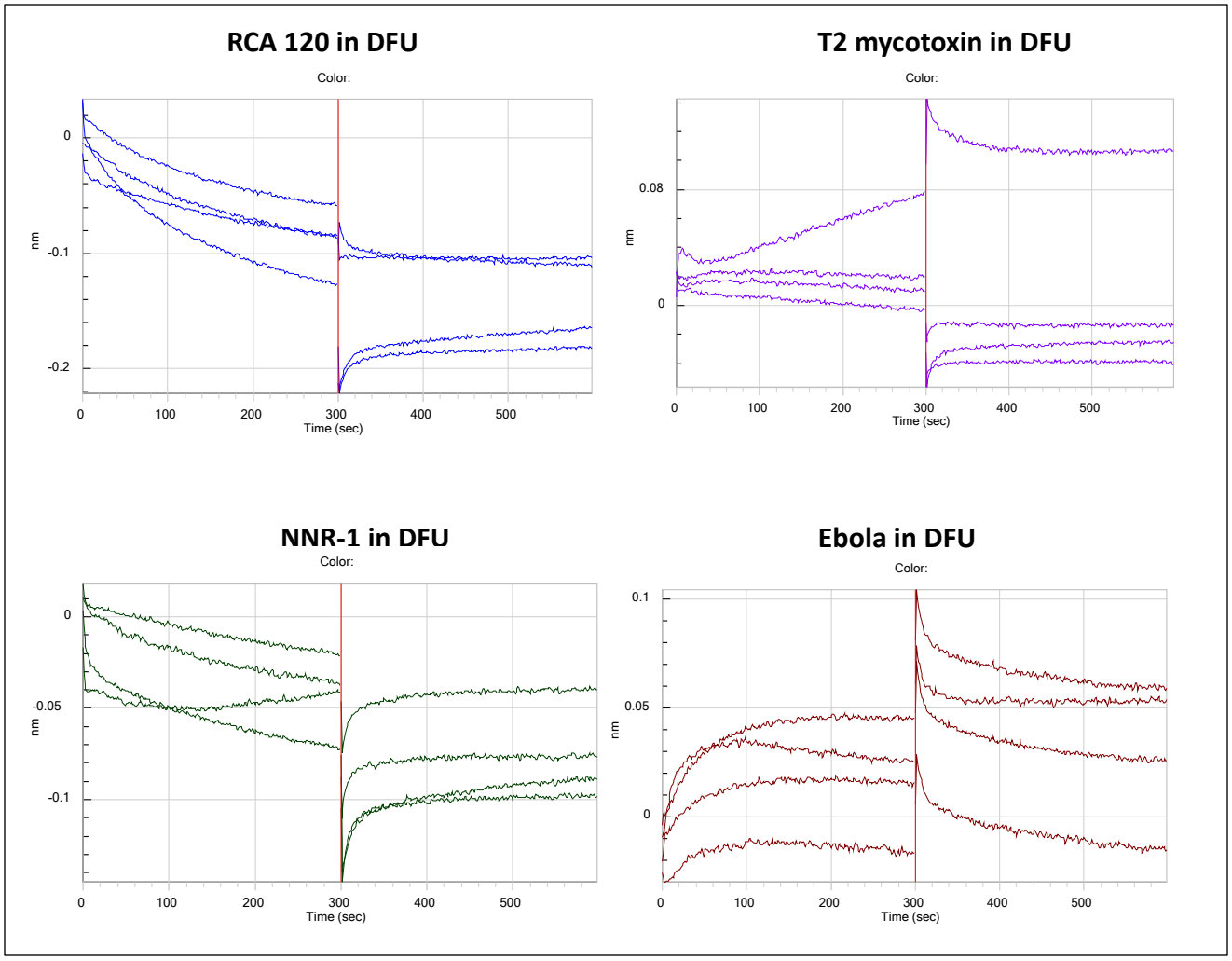

Figure B2. Cocktail 1: RCA 120, T2 mycotoxin, *B. anthracis* NNR-1, and Ebola VLPs in dry filter unit (DFU) matrix.

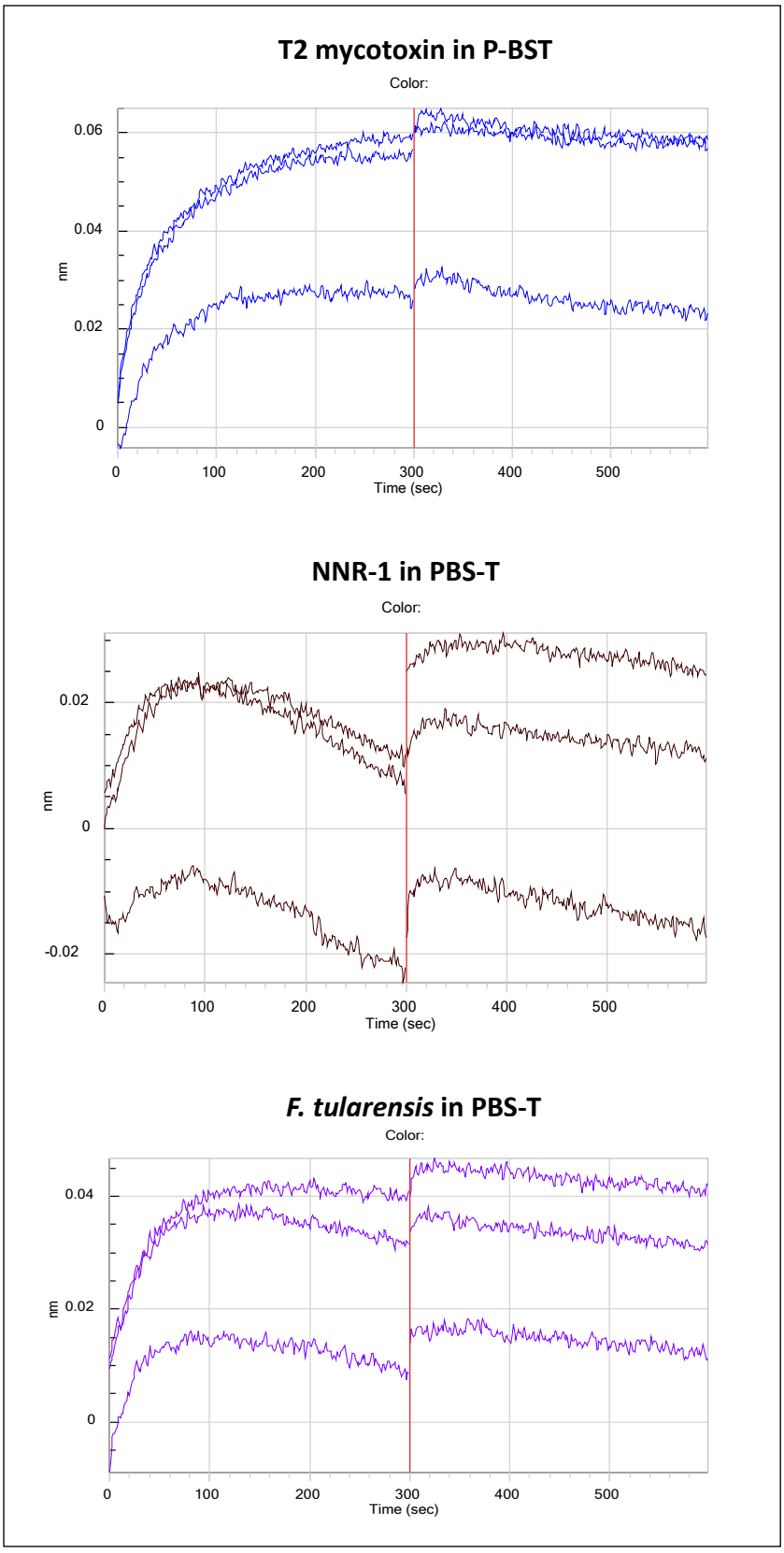

Figure B3. Cocktail 2: T2 mycotoxin, *B. anthracis* NNR-1, and *F. tularensis* in PBS-T.

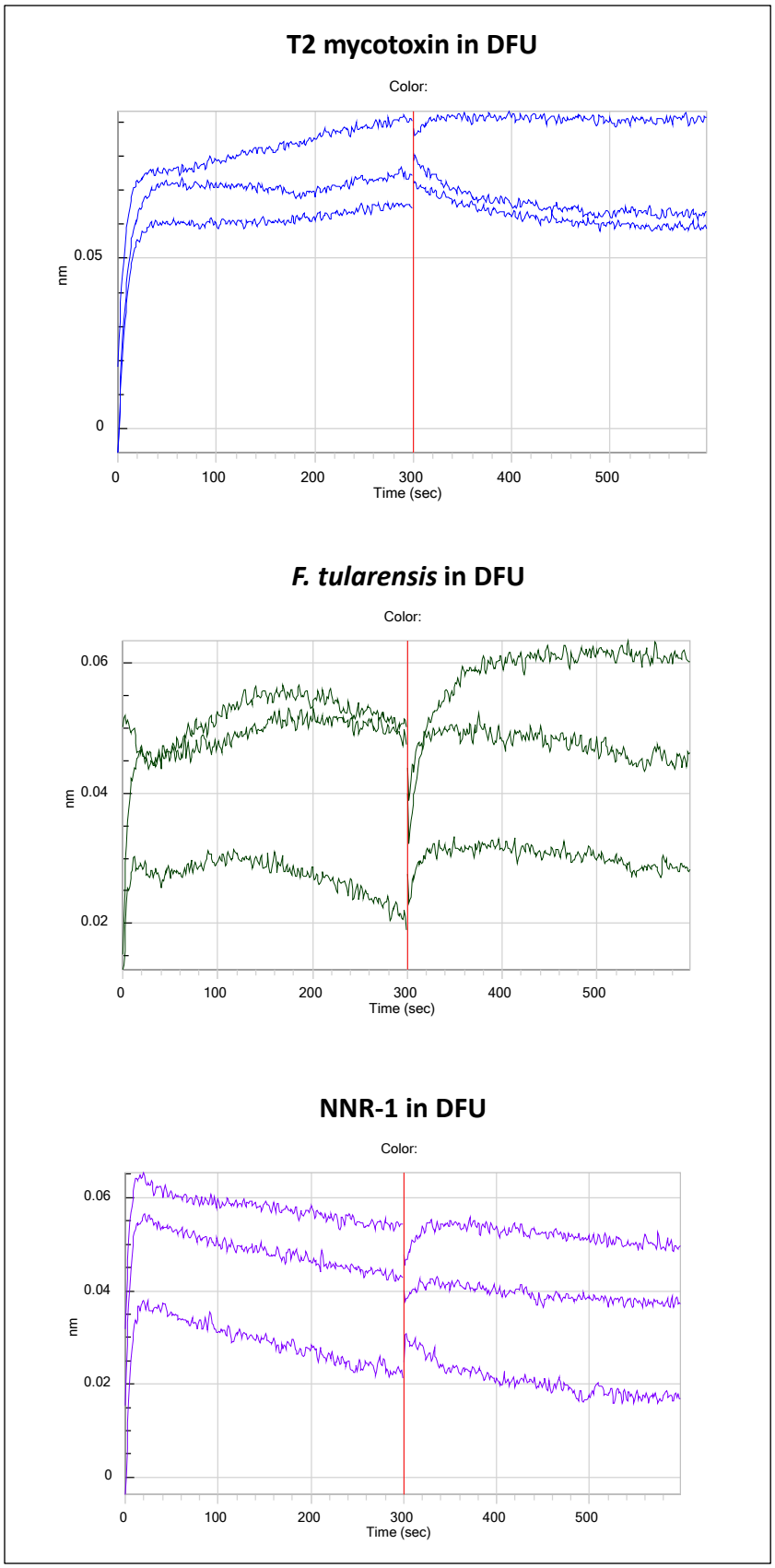

Figure B4. Cocktail 2: T2 mycotoxin, *B. anthracis* NNR-1, and *F. tularensis* in DFU matrix.

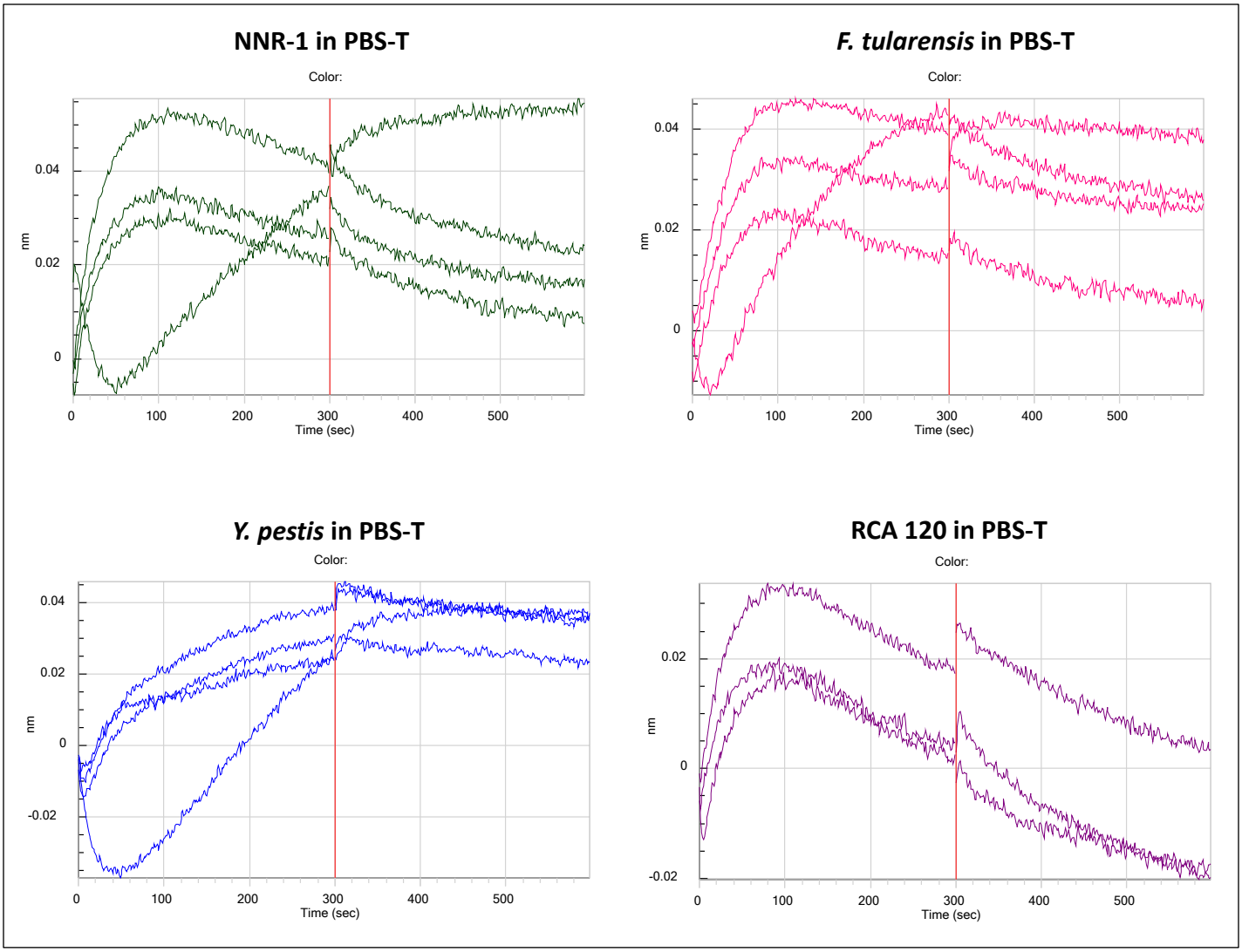

Figure B5. Cocktail 3: *B. anthracis* NNR-1, *F. tularensis*, *Y. pestis*, and RCA 120 in PBS-T.

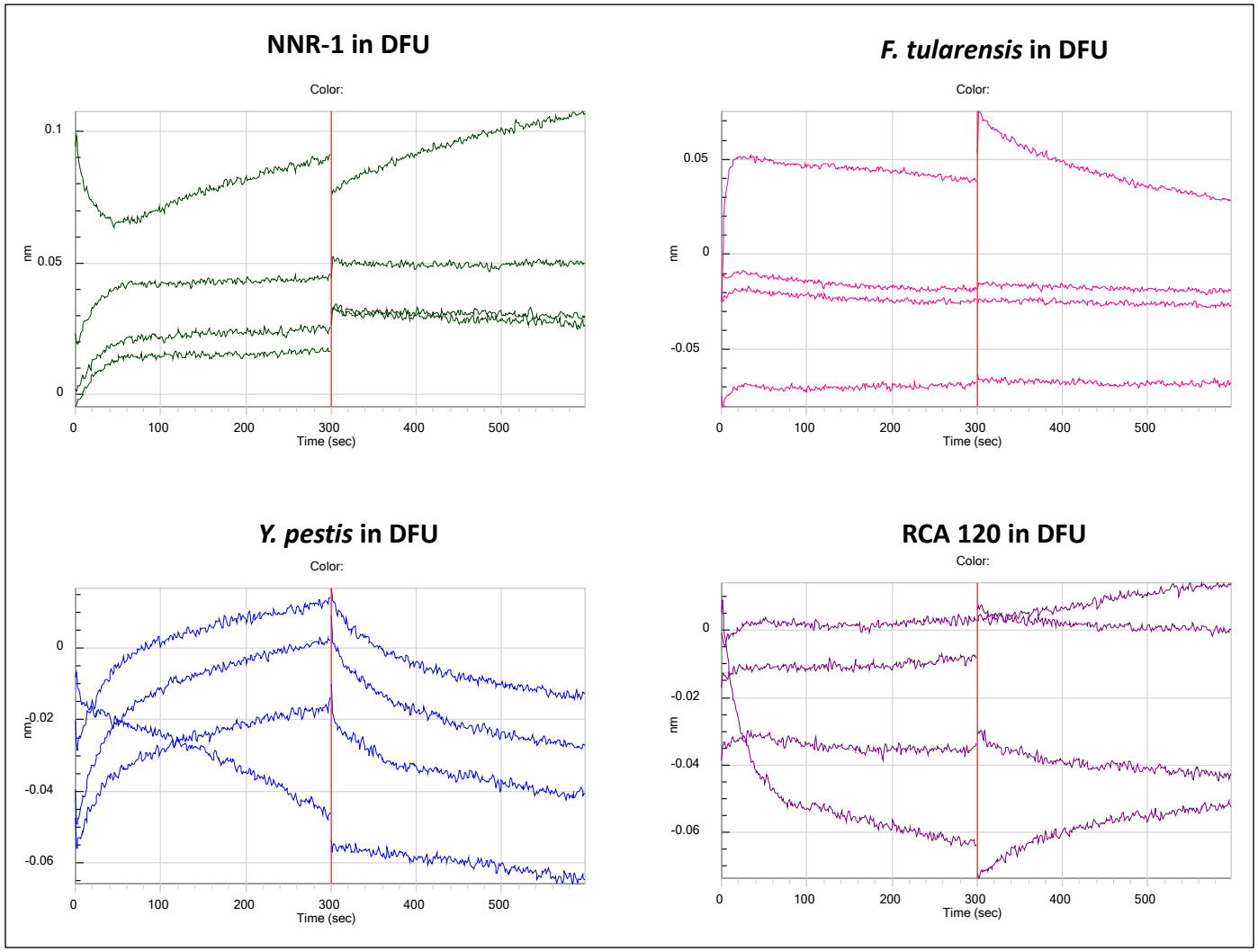

Figure B6. Cocktail 3: *B. anthracis* NNR-1, *F. tularensis*, *Y. pestis*, and RCA 120 in DFU matrix.

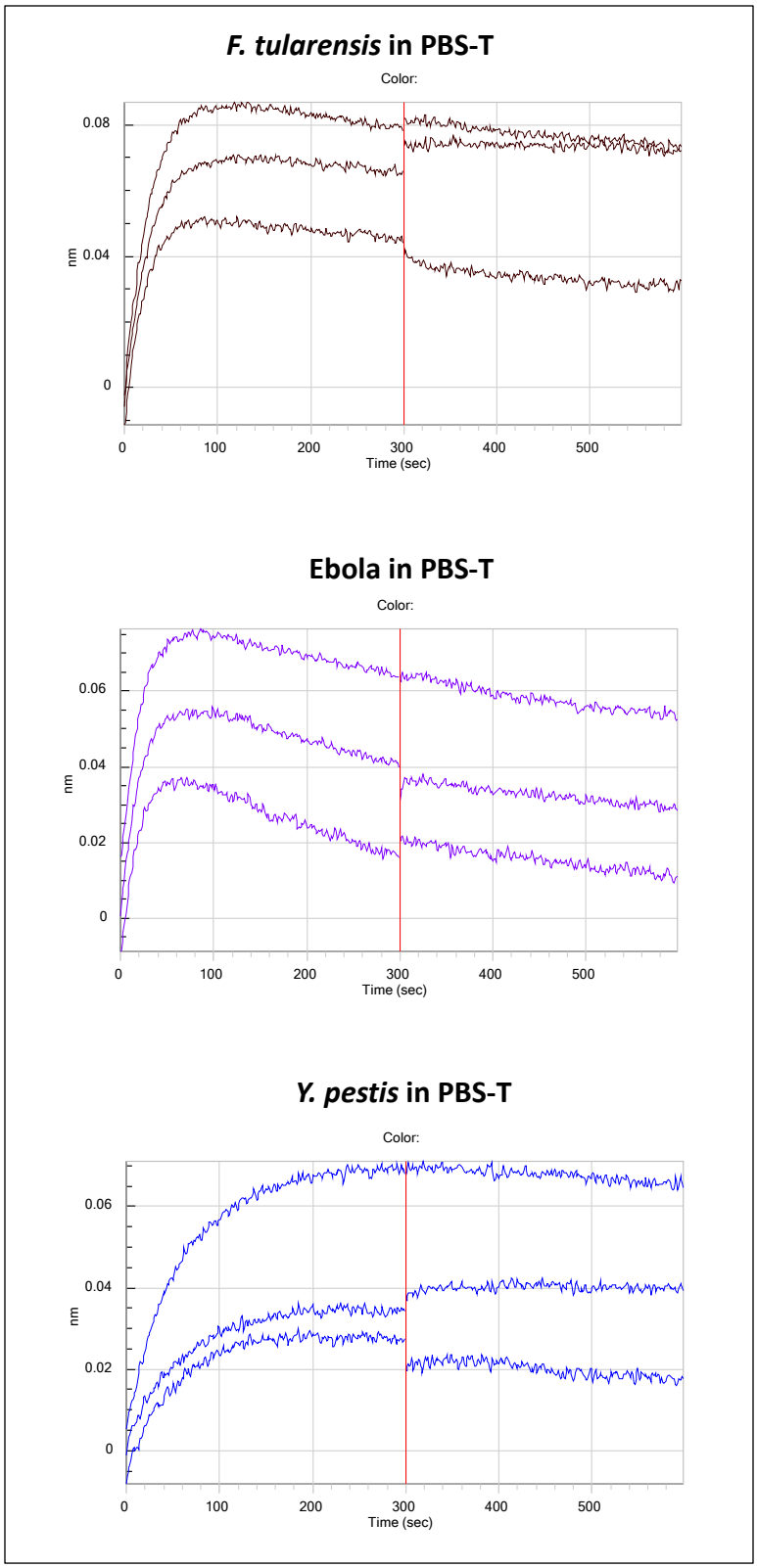

Figure B7. Cocktail 4: *F. tularensis*, *Y. pestis*, and Ebola VLPs in PBS-T.

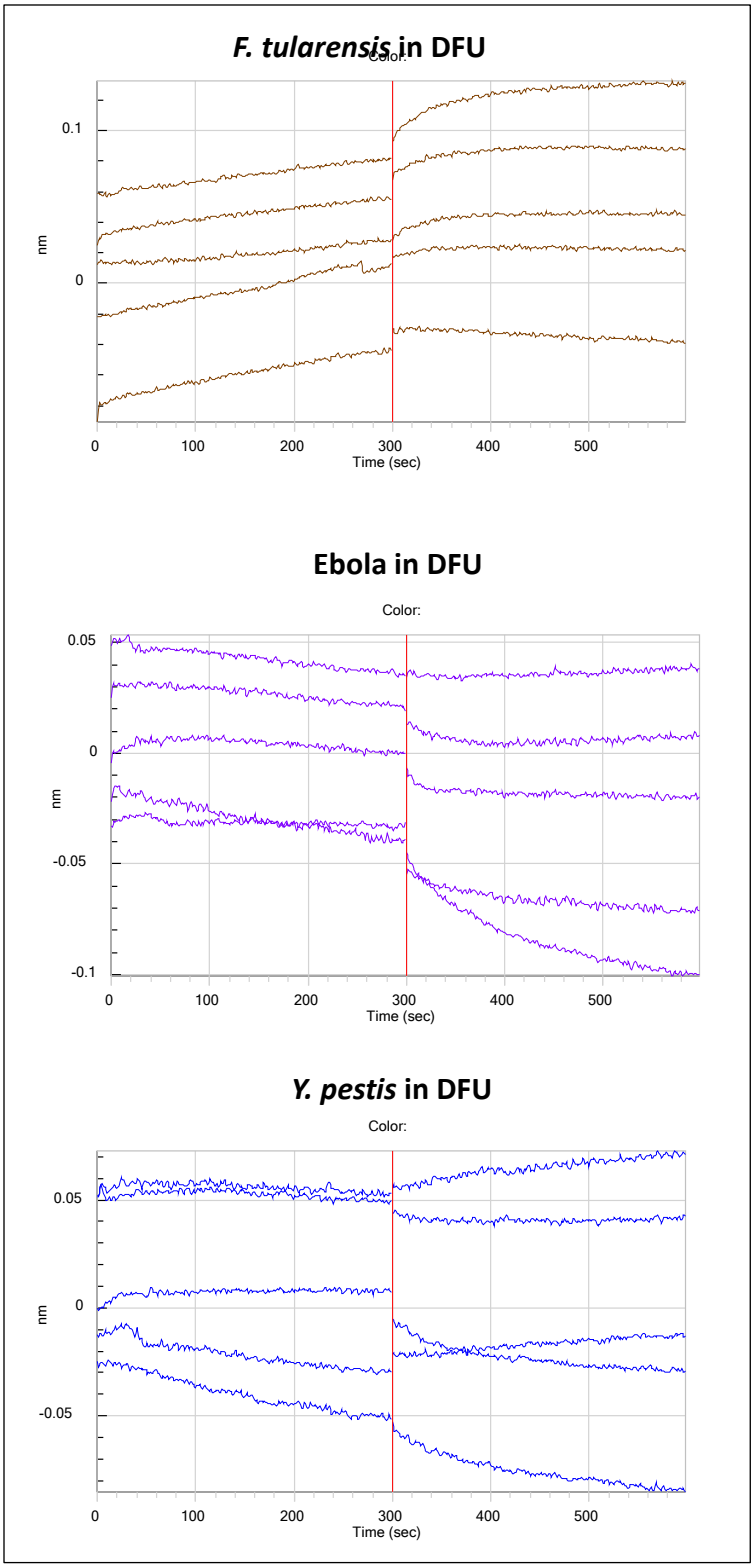

Figure B8. Cocktail 4: *F. tularensis*, *Y. pestis*, and Ebola VLPs in DFU matrix.

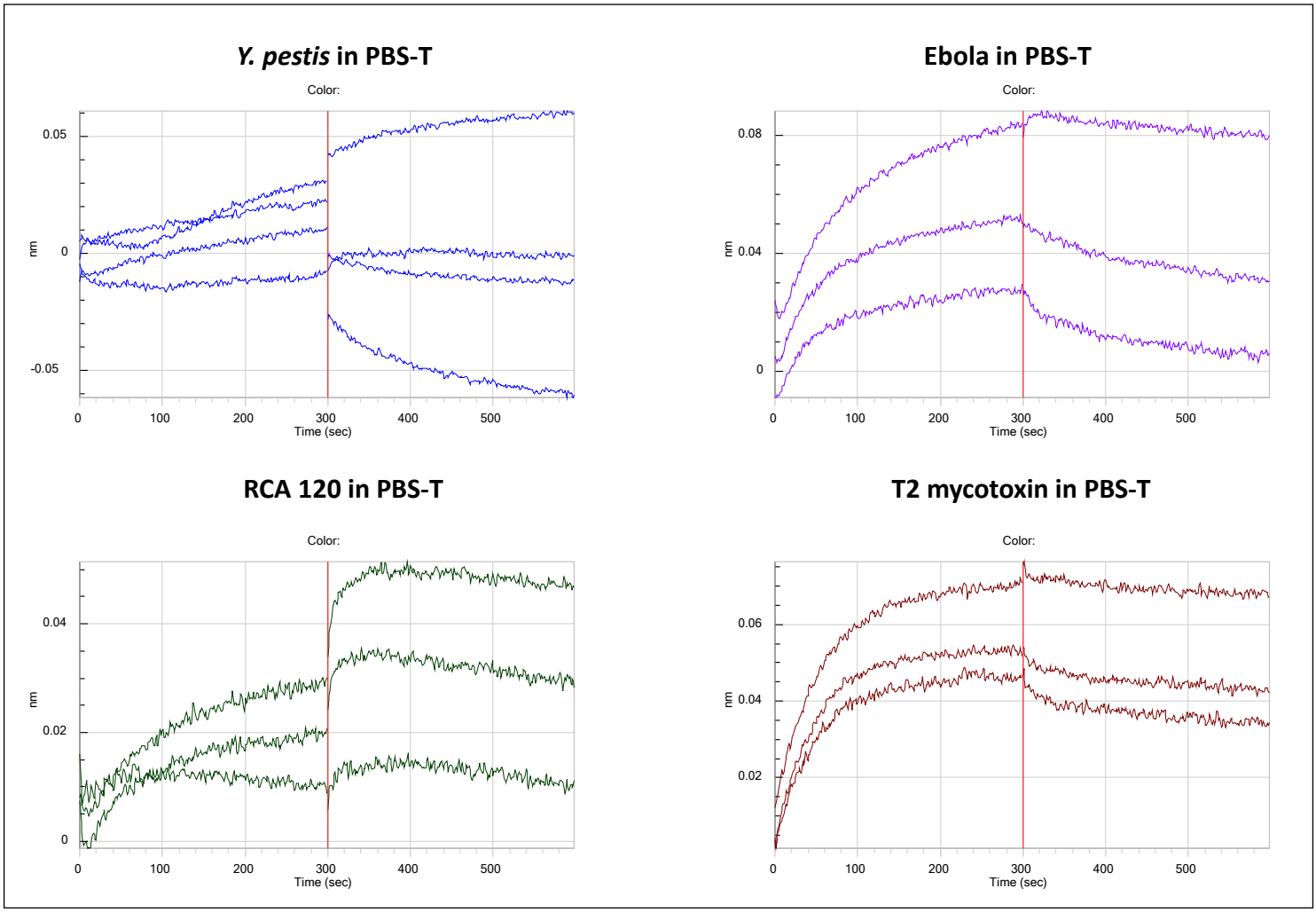

Figure B9. Cocktail 5: *Y. pestis*, Ebola VLPs, RCA 120, and T2 mycotoxin in PBS-T.

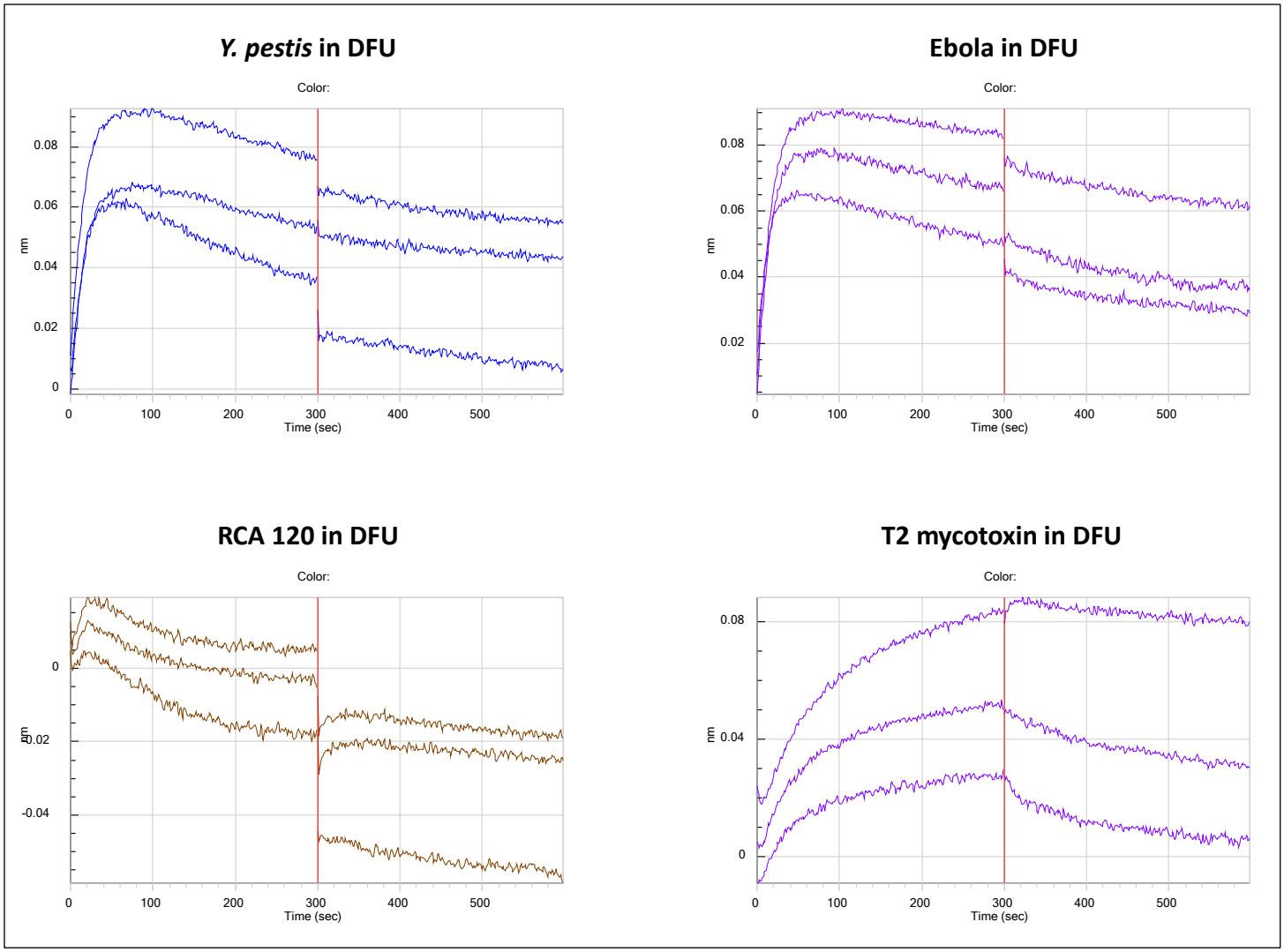

Figure B10. Cocktail 5: *Y. pestis*, Ebola VLPs, RCA 120, and T2 mycotoxin in DFU matrix.

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