U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER

ABERDEEN PROVING GROUND, MD 21010-5424

DEVCOM CBC-TR-1742

DARPA ATP Phase II: Characterization of an Anti-HA33A TXscAb Antibody Produced by the University of Texas

Patricia E. Buckley
Alena M. Calm
Heather Welsh
Roy Thompson
Darrel Menking
James Carney
RESEARCH AND OPERATIONS DIRECTORATE

Candice Warner
Melody Zacharko
EXCET, INC.
Springfield, VA 22150-2519

April 2023

REPORT DOCUMENTATION PAGE

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**	
		START DATE	**END DATE**
XX-04-2023	Final	Sep 2012	Sep 2015

4. TITLE AND SUBTITLE
DARPA ATP Phase II: Characterization of an Anti-HA33A TXscAb Antibody Produced by the University of Texas

5a. CONTRACT NUMBER	**5b. GRANT NUMBER**	**5c. PROGRAM ELEMENT NUMBER**
5d. PROJECT NUMBER	**5e. TASK NUMBER**	**5f. WORK UNIT NUMBER**
BA08DET000/1R3Z11		

6. AUTHOR(S)
Buckley, Patricia E.; Calm, Alena M.; Welsh, Heather; Thompson, Roy; Menking, Darrel; Carney, James (DEVCOM CBC); Warner, Candice; and Zacharko, Melody (Excet)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	**8. PERFORMING ORGANIZATION REPORT NUMBER**
Director, DEVCOM CBC, ATTN: FCDD-CBR-BM, APG, MD 21010-5424 Excet, Inc.; 6225 Brandon Avenue, Suite 360, Springfield VA 22150-2519	DEVCOM CBC-TR-1742

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	**10. SPONSOR/MONITOR'S ACRONYM(S)**	**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**
Defense Advanced Research Projects Agency, 675 North Randolph Street, Arlington, VA 22203-2114	DARPA	

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Statement A. Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES
U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC) was previously known as U.S. Army Edgewood Chemical Biological Center (ECBC).

14. ABSTRACT (LESS THAN 200 WORDS)
The Defense Advanced Research Projects Agency (DARPA; Arlington, VA) Antibody Technology Program (ATP) develops technologies that enhance both the thermal stability and binding affinity of given antibodies. In this study, the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) functioned as an independent testing laboratory to provide technical support on immune reagents as well as assistance in defining the government-supplied antibody–antigen pairs. The goals of this project were to implement standardized methods for characterizing antibodies developed at ECBC with the de novo thermal and binding properties of select reagents to be used in the future by DARPA-funded investigators. Investigators will then use those methods to validate changes in antibody thermal stability and binding affinities. The antibody chosen for enhancement was Bot 56, which detects hemagglutinin-33/A (a part of the botulinum neurotoxin complex type A). The focus of this work was to evaluate the Bot antibody supplied by the DARPA-funded investigator University of Texas (Austin, TX) for affinity and stability enhancements. Results from this study provide standardized parametric data on the antibody properties and performance to support selection of antibody-based reagents that optimize field operational and performance metrics of future detection and diagnostic platforms.

15. SUBJECT TERMS

Antibody	Enzyme-linked immunosorbent assay (ELISA)	Biosurveillance	Quality
Testing	Hemagglutinin-33/A (HA33A)	Bot 56	Botulinum neurotoxin

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**
a. REPORT	**b. ABSTRACT**	**C. THIS PAGE**		
U	U	U	UU	34

19a. NAME OF RESPONSIBLE PERSON	**19b. PHONE NUMBER** *(Include area code)*
Renu B. Rastogi	(410) 436-7545

STANDARD FORM 298 (REV. 5/2020)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized under project no. BA08DET000/1R3Z11. The work was started in September 2012 and completed in September 2015. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

Acknowledgments

The authors thank Kelley Betts (Excet; Springfield, VA) for assistance in editing and preparing this report.

Blank

CONTENTS

FIGURES

TABLES

DARPA ATP PHASE II: CHARACTERIZATION OF AN ANTI-HA33A TXscAb ANTIBODY PRODUCED BY THE UNIVERSITY OF TEXAS

1. INTRODUCTION

The Joint Product Management Office for Biosurveillance (JPMO BSV; now the Joint Program Executive Office for Chemical, Biological, Radiological and Nuclear Defense; Aberdeen Proving Ground, MD) instituted a quality program to standardize test methods to more fully characterize and compare the physical and functional properties of antibody reagents in its repository. Production methods for antibodies used in detection devices have drastically changed over time. Animal models (the most common method) produced polyclonal antibodies that lacked antigen binding specificity, and the resulting antibody affinity depended on the individual animal. Development of the monoclonal antibody improved specificity but still required the use of large numbers of animals. With the advent of hybridoma cell culture production, larger quantities of high-activity antibodies were produced, and existing cell lines could be panned for higher-affinity antibodies. Recombinant methods greatly increased researchers’ ability to produce more specific antibodies with antigen-binding fragments (Fabs), single-chain variable fragments (scFvs), and single-domain antibodies. A variety of recombinant production systems, ranging from basic bacteria, yeasts, and filamentous fungi to insect cell lines, mammalian cells, and transgenic plants and animals, are being used (*1*).

The Defense Advanced Research Projects Agency (DARPA; Arlington, VA) Antibody Technology Program (ATP) focused on the development of technologies that enhanced both the thermal stability and the binding affinity of a given antibody. Increased thermal stability would eliminate the need for cold storage and increase the usability of antibodies in the types of harsh conditions experienced by troops in the field. Increasing binding affinity would allow for the production of multiplex sensors that could detect an increased variety of antigens. The U.S. Army Edgewood Chemical Biological Center (ECBC; now the U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) functioned as an independent testing laboratory for this program and provided specific technical support on immune reagents, as well as assistance in defining the government-supplied antibody–antigen pairs. Standardized methods developed at ECBC for characterizing antibodies were used to validate the changes in antibody thermal stability and binding affinity achieved by the DARPA investigators. Combinatorial approaches to antibody enhancement are random and may lead to fortuitous improvements in stability or affinity; therefore, the strategies for ATP enhancements were directed, so they could be transferable to other antibody molecules.

The primary objectives for the Phase I ATP were to develop and demonstrate strategies that independently modulated antibody stability and affinity in an antibody molecule provided by the government. The Phase II goals were to modify an antibody using the Phase I techniques and produce 2 g of a single protein that yielded a 100-fold increase in affinity and a 10 °C increase in the melting temperature of a provided protein. The focus of this work was the evaluation of the anti-botulinum toxin (Bot) antibody for affinity and stability enhancements. The Bot antibody was supplied by the DARPA-funded investigator University of Texas (Austin, TX).

2. MATERIALS AND METHODS

2.1 Antibody and Antigen

2.1.1 Choice of Antibody

The Critical Reagents Program (CRP; now known as the Defense Biological Product Assurance Office; Fort Detrick, MD) collection contains many polyclonal and monoclonal Bot antibodies. For this project, the recombinant Bot 56 antibody that detects hemagglutinin-33/A (HA33A), a part of the botulinum neurotoxin complex Type A, was chosen.

2.1.2 Choice of Antigen

The antigen, HA33A from *Clostridium botulinum*, is resistant to protease digestion and likely protects the neurotoxin from activation. Recombinant HA33A plasmids were transformed via electroporation into BL21(DE3) *Escherichia coli* cells (MilliporeSigma; Burlington, MA). A single colony was used to inoculate 1 mL of Luria broth (LB) (Thermo Fisher Scientific; Waltham, MA) containing 100 µg/mL kanamycin (kan) (Thermo Fisher Scientific) and incubated overnight at 37 °C and 300 rpm. In the morning, the culture was scaled up to 50 mL of LB and incubated at 37 °C and 225 rpm until it reached log phase. The log-phase culture was scaled up by adding 10 mL to 500 mL of terrific broth media (MilliporeSigma) containing 100 µg/mL kan. The cultures were grown for 20 h, the cell paste was collected via centrifugation, and the cells were mechanically lysed at 20,000 psi using an M110P microfluidizer (Microfluidics; Westwood, MA). HA33A was purified over a nickel affinity column on an ÄKTAxpress chromatography system (Cytiva Life Sciences; Marlborough, MA) and desalted into 50 mM citric acid (pH 4.0). The purified protein was minimally biotinylated using an EZ-Link sulfo-NHS-biotinylation kit (Thermo Fisher Scientific) to allow for the use of streptavidin–horseradish peroxidase (strep–HRP) in the enzyme-linked immunosorbent assay (ELISA).

2.1.3 Bot 56 Antibody Modification

The Bot 56 antibody was modified by the University of Texas as previously described (*2*). Briefly, test personnel “supercharged” the antibodies by increasing the net charge of amino acids on the protein surface. The Rosetta macromolecule modeling suite (Rosetta Commons; https://www.rosettacommons.org/) was used to predict the energetic consequences of the substitutions. The modifications increased resistance to aggregation and could enable reversible unfolding. The modified antibody is designated as TXscAb in this report.

2.2 Test Methods

The parametric tests that were established and standardized using the MS2 scFv antibody during DARPA ATP Phase I (*3*) were applied in this study.

2.2.1 Antibody Concentration Measurement

TXscAb and Bot 56 concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). This instrument provides an absorbance reading at 280 nm (A_{280}) for the sample. To determine an accurate concentration for each sample, the average of the A_{280} data was divided by the extinction coefficients for scFv antibodies (1.66) and Bot 56 Fabs (1.77). Each reading required a 2 µL sample, which was placed on the sample pedestal. The instrument was blanked using phosphate-buffered saline (PBS; MilliporeSigma), and readings were taken in triplicate. Bovine γ-globulin (BGG; Bio-Rad; Hercules, CA) was also tested as a positive control to validate instrument operation.

2.2.2 Molecular Weight and Purity Measurements

An Experion automated electrophoresis system (Bio-Rad) was used to determine the molecular weight and purity of both TXscAb and Bot 56. In conjunction with the Experion reagents, electrophoresis station, and software, the microfluidic chip is designed to accomplish separation, staining, destaining, detection, and basic data analysis. Sample purity was determined by calculating the percent mass of the separated proteins in a sample. For analysis, each antibody was standardized to a final concentration of 1 mg/mL by dilution in PBS. The control (BGG) and antibody samples were then processed using the validated procedure specified in the instruction manual provided with the Experion electrophoresis system (*4*). Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. The sample was reduced with dithiothreitol (MilliporeSigma) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip. The instrument was operated via the Experion software; all samples were tested in triplicate alongside one sample of the control (BGG) and Pro260 ladder (Bio-Rad). Analysis was also performed using the Experion software.

2.2.3 Protein Behavior Measurement Using Dynamic Light Scattering (DLS)

DLS was used to evaluate how the proteins behaved in solution. In DLS, three tests are performed to determine protein uniformity. Polydispersity is a measure of the size distribution of particles in solution; for protein analysis, polydispersion of less than 20% is considered monodispersion. Hydrodynamic radius and molecular weight of the sample are displayed by two graphs. The correlation graph indicates the relative size of the particle, and the steepness of the line indicates the level of monodispersion of the sample. The regularization graph derived from the data shows the hydrodynamic radius, percent mass, and molecular weight. Prediction algorithms are used by the DLS software to produce this range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the antibody, along with the control (BSA), were placed into a 384 well quartz plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at 239*g* to remove trapped air bubbles from the samples. Mineral oil (MilliporeSigma) was applied to the top of each sample to prevent sample evaporation, and the plate was then placed into a temperature-controlled DynaPro plate reader (Wyatt Technology Corporation). Each well was scanned 10 times for 5 s each at 25 °C, and results were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight using Dynamics software (Wyatt Technology Corporation). The results for three wells were averaged and reported.

2.2.4 Thermal Stability Measurement

Differential scanning calorimetry (DSC) was used to get a quantitative melting temperature (T_m) for each of the antibody proteins. The T_m is determined to predict results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C predicts that antibody activity after the thermal stress test will remain above 50%. A T_m below 70 °C predicts, at minimum, a 50% decrease in antibody activity after the thermal stress test. For DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS, pH 7.4. Samples were degassed for 5 min before analysis and injected into the sample cell of a MicroCal VP-DSC system (North Hampton, MA). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all experiments. The samples were scanned from 15 to 100 °C at a rate of 60 °C/h in duplicate. The transition midpoint of the protein was determined by analysis of the data using Origin 7.0 software (OriginLab; Northampton, MA).

2.2.5 Thermal Stress Test

All samples were diluted to a 1 mg/mL concentration before heat was applied to negate protective effects due to concentration. Antibodies were diluted to 1 mg/mL in 1× PBS and divided among five tubes. One aliquot was kept on ice for the duration of the experiment and marked "time 0". The remaining four aliquots were heated to 75 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. ELISAs were then conducted to test the samples for activity.

2.2.6 ELISA

Following the thermal stress testing, ELISAs were performed in triplicate using the standard capture assay technique. Each antibody sample was diluted to 20 µg/mL in PBS. A twofold serial dilution was performed across each Nunc Maxisorp 96 well plate (Thermo Fisher Scientific). Samples were then incubated at 4 °C overnight. In the morning, each plate was washed in 1× wash buffer (KPL; Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (MDS Analytical Technologies; Sunnyvale, CA) and then blocked with 1× milk diluent block (MDB; KPL) for 30 min at 37 °C. The plates were then washed again, and 100 µL of 0.125 µg/mL biotinylated HA33A antigen was added to each well. The plates were then incubated for 1 h at room temperature (RT). Strep–HRP (KPL) was diluted to 0.1 µg/mL in 1× MDB, 100 µL of which was added to each well and incubated at RT for 1 h. After washing, 100 µL of RT 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) one-component HRP substrate (KPL) was added to each well. After 9 min at RT, the optical density at the 405 nm light wavelength was determined using a Synergy H4 hybrid multi-mode microplate reader (BioTek; Winooski, VT). Data analysis was performed using Prism GraphPad software (version 5.00 for Windows; GraphPad Software; San Diego, CA).

2.2.7 SPR

SPR is used to determine the kinetic parameters of an antibody–antigen interaction. This rapid methodology monitors biomolecular interactions through the excitation of surface plasmons that results when polarized light is shined through a prism on a sensor chip with a thin metal film coating. The metal film acts as a mirror and reflects the light, and when the angle of light through the prism is changed, the intensity of the reflected light can be monitored and differences in intensity recorded. While the refractive index at the prism side of the chip does not change, the refractive index in the immediate vicinity of the metal surface will change when accumulated mass (bound proteins) adsorbs onto the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this shift of the SPR angle provides information on the kinetics of the protein adsorption on the surface. The SPR software then provides an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions and calculates the overall affinity constant (K_D) between the antibody and antigen.

2.2.7.1 Thermostability Testing

Using the Biacore T200 SPR system (Cytiva), 6500 response units (RUs) of HA33A were tethered to one flow cell of a CM5 chip (Cytiva) using standard amine coupling chemistry. After thermal stress testing, samples were centrifuged at 5 °C and 2000*g* for 5 min. The analyte was then run at a rate of 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated antibody at 400, 350, 300, 250, 200, 150, 100, and 50 nM and plotting the respective maximum relative fluorescence units (R_{Max}). Unheated and heated samples were then diluted at ratios of 1:90 and 1:180 in order for the time 0 control points to fall on the linear calibration curve. All samples were run in triplicate. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Biacore Concentration Analysis software (Cytiva) was used for data collection, and the active concentration of heated sample was recorded. The running buffer used for this experiment was Biacore 1× HBS-EP buffer (Cytiva).

2.2.7.2 Kinetic Analysis

Using the Biacore T200 SPR system and 1× HBS-EP running buffer, 102 RUs of HA33A were tethered to a CM5 chip (GE Healthcare) using standard amine coupling chemistry. TXscAb was injected across the chip's surface for 120 s at a flow rate of 75 µL/min with a 900 s dissociation at 60, 20, 6.67, and 2.2 nM and 700 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 30 µL/min with a 60 s stabilization period. Data were analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Antibody Concentration Measurements

3.1.1 TXscAb

TXscAb results were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings were as follows:

Replicate 1: 2.062,
Replicate 2: 2.059, and
Replicate 3: 1.977.

An average was determined from the A_{280} results (the spectrophotometer software automatically divided by the extinction coefficient). The reported concentration was 2.03 mg/mL.

3.1.2 Bot 56

Bot 56 results were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings were as follows:

Replicate 1: 7.60,
Replicate 2: 7.65, and
Replicate 3: 7.75.

An average was determined from the A_{280} results (the spectrophotometer software automatically divided by the extinction coefficient). The reported concentration was 7.7 mg/mL.

3.2 Molecular Weight and Purity Measurements

3.2.1 TXscAb Molecular Weight and Purity

The molecular weight of TXscAb was determined using the Experion Pro260 analysis kit, as shown in Figure 1. The thick band at the top of the second lane corresponds to the single-chain antibody fragment. According to the software, the antibody was 87.4% pure, and the heavy chain weighed 48.6 kDa.

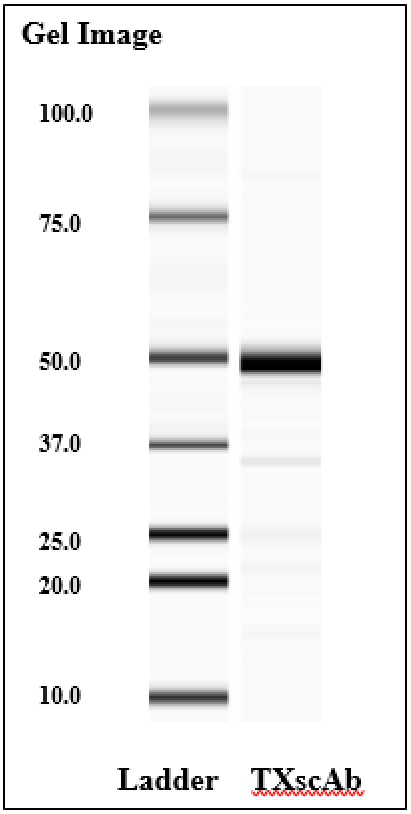


Figure 1. Molecular weight and purity of TXscAb.

3.2.2 Bot 56 Molecular Weight and Purity

The molecular weight of Bot 56 was measured in the same manner as TXscAb (Section 3.2.1). Figure 2 shows a digital gel of Bot 56 produced by the Experion Pro260 system. The thick band at the top of the right lane corresponds to the Bot 56 heavy chain with a molecular weight of 30.4 kDa; the thinner band at the bottom of the second lane corresponds to the light chain and amolecular weight of 27.5 kDa. The purity was measured at 97.3%.

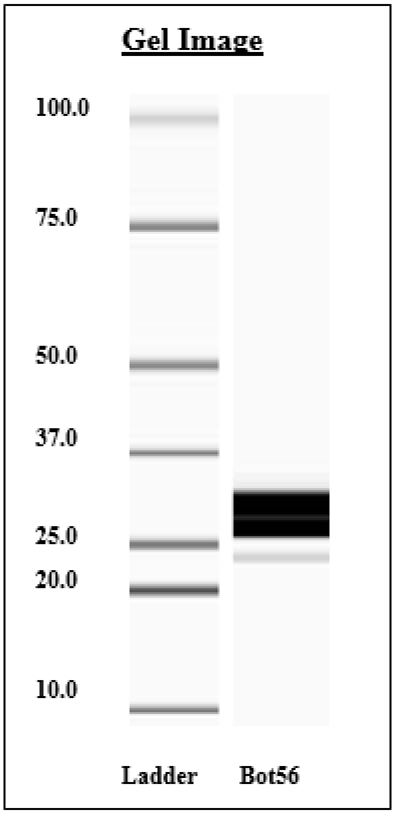


Figure 2. Molecular weight and purity of Bot 56.

3.3 Protein Behavior by DLS

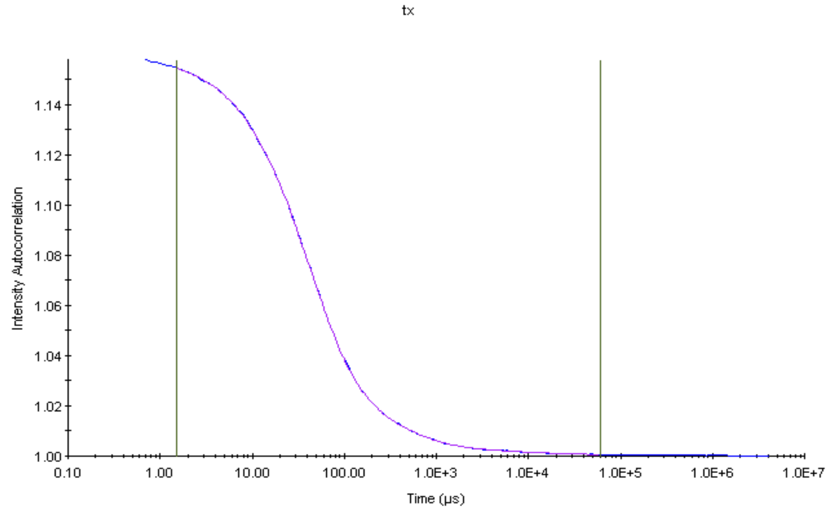
3.3.1 TXscAb Protein Behavior

TXscAb was analyzed in triplicate using the DynaPro plate reader. The radius of TXscAb was determined to be 7.5 nM, and it had a polydispersity of 51.4% (Table 1). Figure 3 contains a representative correlation graph and regularization graph for TXscAb. The correlation graph (panel A) depicts a sigmoidal curve indicative of a valid size distribution. The regularization graph (panel B) illustrates the monodispersity found in both samples. Table 1 shows the raw data produced for each replicate. Because 99.0% of the mass displayed high polydispersity, this sample preparation was considered to be polydisperse.

Table 1. Features of TXscAb in Solution

Replicate	Radius (nM)	Polydispersity (%)
1	8.59	64.6
2	6.95	46.2
3	7.23	47.7
4	8.19	57.0
5	6.55	41.3
Average	7.50	51.4

A. Correlation Graph of TXscAb.



B. Regularization Graph of TXscAb

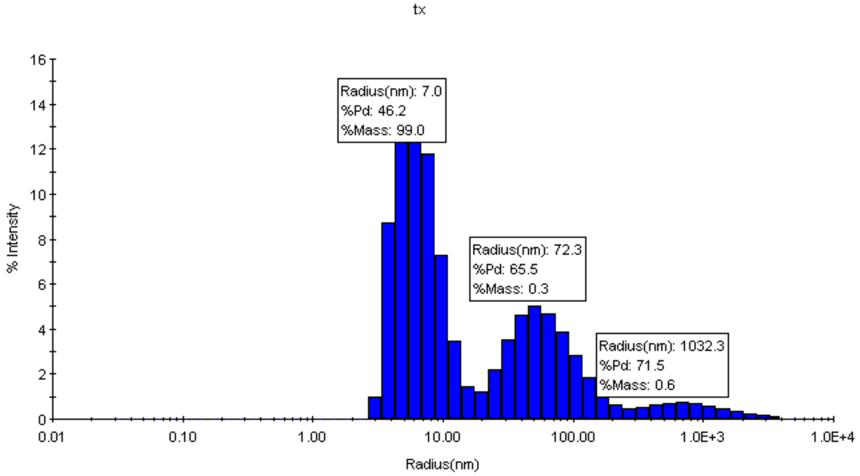


Figure 3. Radius and polydispersity (Pd) representation of TXscAb. (A) Correlation graph and (B) regularization graph of the thermostable TXscAb.

3.3.2 Bot 56 Protein Behavior

Bot 56 was analyzed in the same manner as TXscAb (Section 3.3.1). The radius of Bot 56 was determined to be 3.3 nM, and it had a polydispersity of 12.0. Figure 4 contains a representative correlation graph and regularization graph for Bot 56. The correlation graph (panel A) depicts a sigmoidal curve indicative of a valid size distribution. The regularization graph (panel B) illustrates the monodispersity identified in both samples. Table 2 shows the raw data produced for each replicate. Because 100% of the mass displayed favorable polydispersity and hydrodynamic radius, the sample preparation was considered to be monodisperse.

Table 2. Bot 56 Protein Behavior Raw Data

Replicate	**Radius (nM)**	**Polydispersity (%)**
1	3.3	11.7
2	3.3	14.2
3	3.2	11.5
4	3.3	11.6
5	3.2	11.1
Average	3.3	12.0

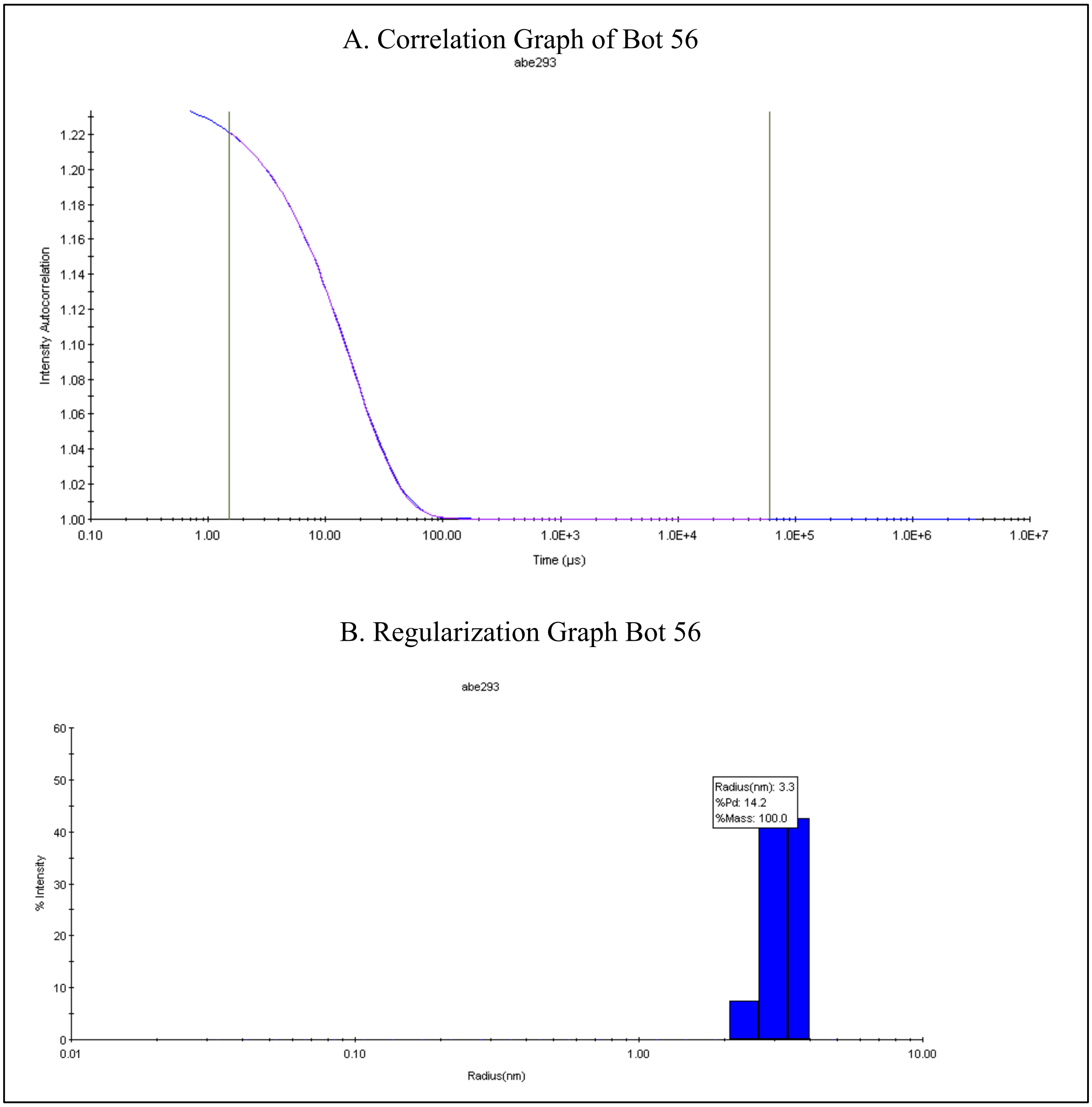


Figure 4. Radius and polydispersity (Pd) representation of Bot 56. (A) Correlation graph and (B) regularization graph of Bot 56 used for determining the radius and polydispersity of the sample.

3.4 DSC

3.4.1 TXscAb Melting Temperature

TXscAb was read in duplicate on the MicroCal VP-DSC system. As shown in Figure 5, the peak T_m values were 77.18 °C for replicate 1 and 77.02 °C for replicate 2.

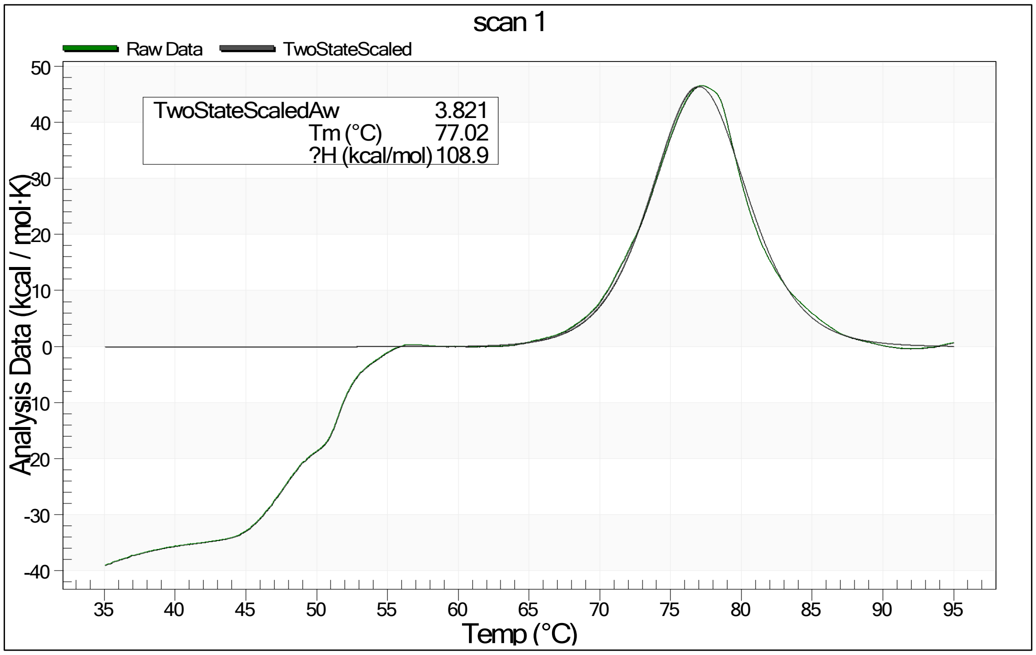


Figure 5. Transition midpoint curve for TXscAb. Average T_m was calculated to be 77.1 °C.

3.4.2 Bot 56 Melting Temperature

Bot 56 was read on the MicroCal VP-DSC system. As shown in Figure 6, the peak T_m was 82.09137 °C.

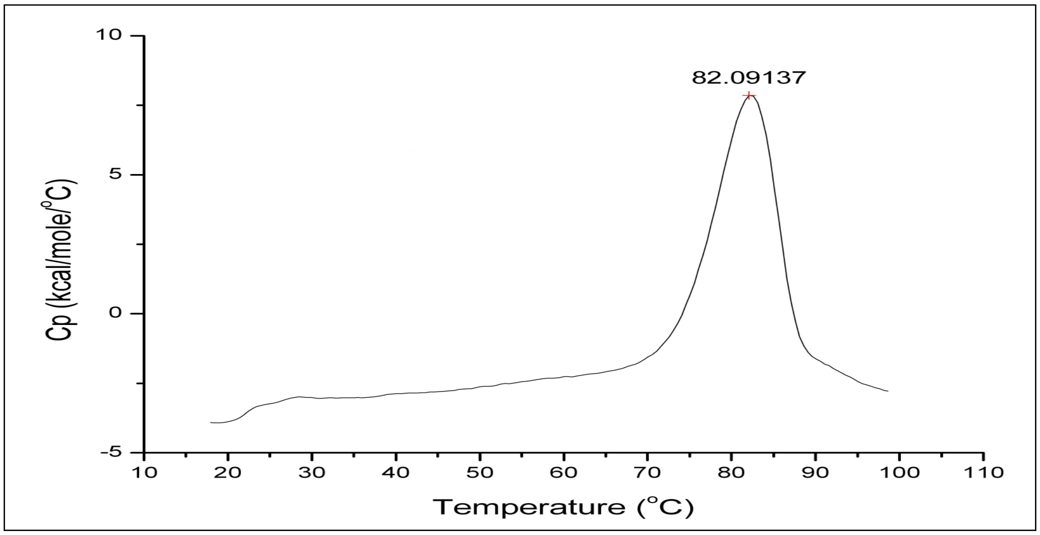


Figure 6. Transition midpoint curve for Bot 56. T_m was calculated to be 82.1 °C.

3.5 Post-Temperature-Stress ELISA

3.5.1 TXscAb ELISA

ELISA was used to test the functional interaction of antibody and antigen after thermal stress at 75 °C. The ELISA data (Figure 7) show that when TXscAb was heated to 75 °C, it maintained all activity across all time periods of thermal stress. The curve in Figure 7 shows antibody activity for different time points at 75 °C as a function of the antigen concentration supplied. The area under the curve for each of the different time points at 75 °C was calculated, averaged, and graphed to depict how TXscAb reacted over time to thermal stress. This graph (Figure 7) illustrates that the antibody remained functionally capable of binding to antigen after a 60 min exposure to 75 °C.

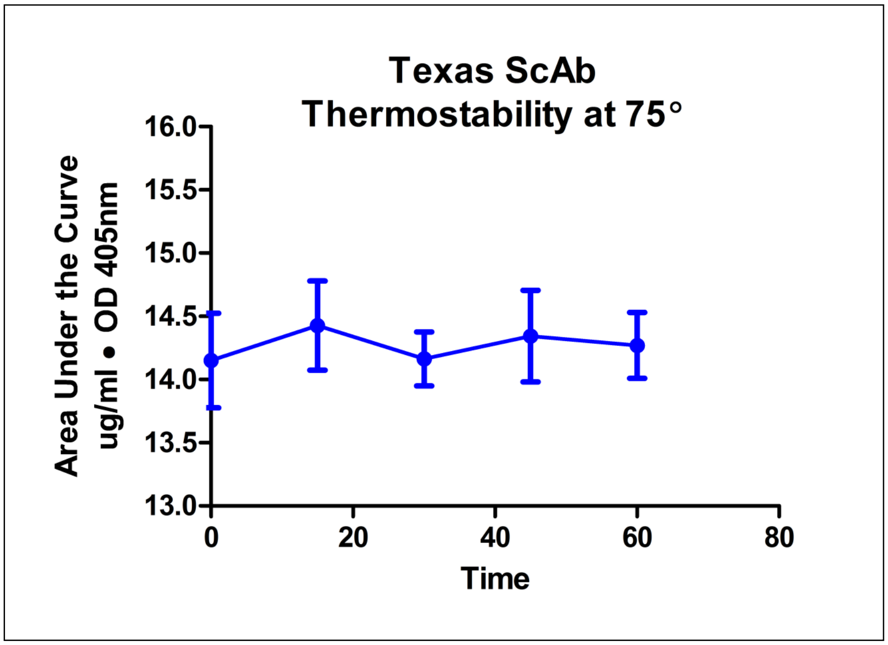


Figure 7. Thermostability of TXscAb ELISA. Area under the curve analysis depicts the effect of thermal stress.

3.5.2 Bot 56 ELISA

ELISA was used to test the functional interaction of Bot 56 as described in Section 3.5.1. The ELISA data (Figure 8) show that when Bot 56 was heated to 75 °C, it maintained all activity for an approximately 30 min exposure.

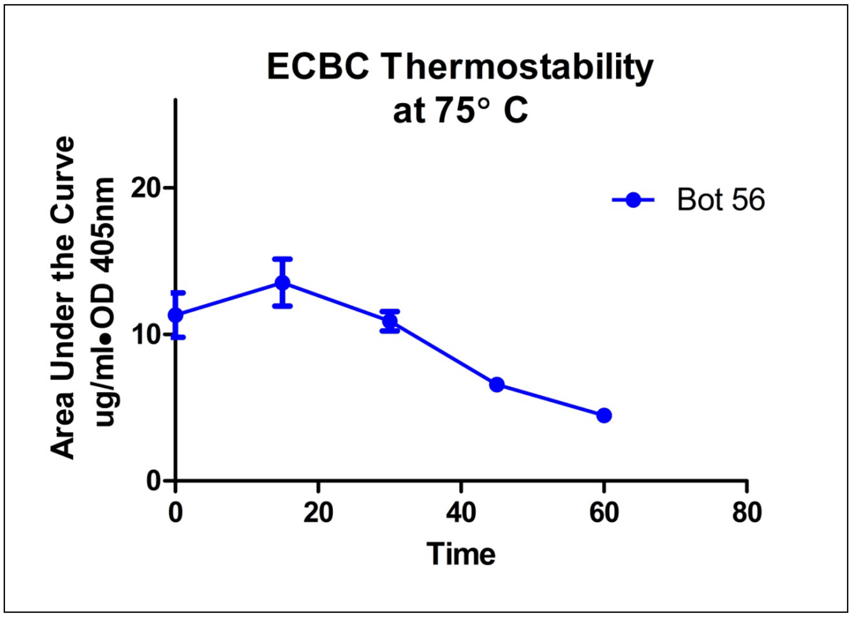


Figure 8. Thermostability of Bot 56 ELISA. Area under the curve analysis depicts the effect of thermal stress.

3.6 SPR

3.6.1 Thermostability Testing by SPR

The functional binding between TXscAb and the antigen was also assessed by SPR after the samples were heated to 75 °C for variable time periods. Five tubes of TXscAb diluted to 1 mg/mL in 1× PBS were prepared and heated to 75 °C for 15, 30, 45, and 60 min and were then quenched on ice. The activity of each sample was compared to a reference standard of unheated sample using a Biacore T200 SPR system (Figures 9 and 10). The activity curve (Figure 9) shows TXscAb retained more than 90% of its activity at 75 °C for 60 min. This indicates TXscAb has greater heat resistance than Bot 56 (Figure 10), which only retained 23% of its activity after 60 min.

Stability was maintained as presented in Table 3.

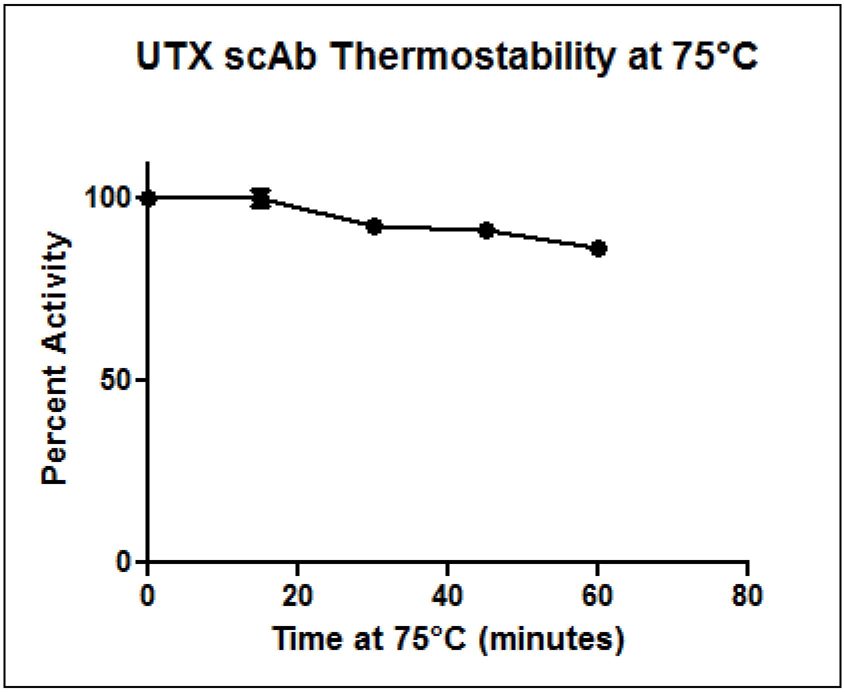


Figure 9. Thermostability of TXscAb. TXscAb maintained more than 90% of its activity after being heated to 75 ºC for 60 min.

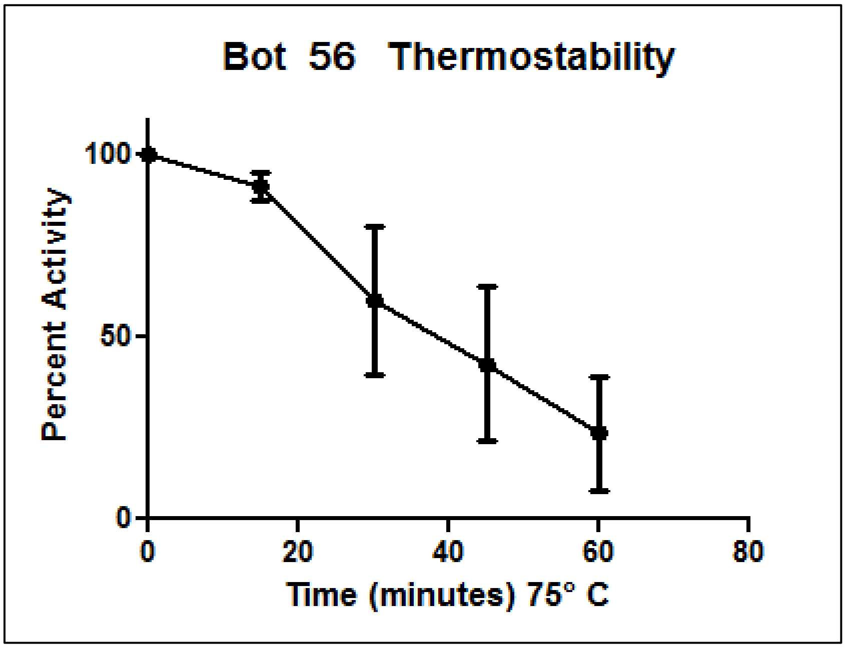


Figure 10. Thermostability of TXscAb compared to Bot 56 by SPR.

Table 3. Stability

Time (min)	Stability (%)	
	TXscAb	**Bot 56**
15	100	92
30	94	60
45	94	42
60	85	23

3.6.2 Kinetic Analysis by SPR

Kinetic analysis of TXscAb binding to the HA33A antigen was performed as a direct-binding SPR experiment on the Biacore T200 SPR system; results are presented in Figure 11. Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using Biacore T200 software (Cytiva). The K_D for TXscAb was determined to be 34 nM. Similar experiments were conducted using the original Bot 56 antibody; results are presented in Figure 12. The K_D for Bot 56 was determined to be 4.19 nM. Based on these results, it can be concluded that the University of Texas did not provide an antibody that met the 100-fold improvement threshold.

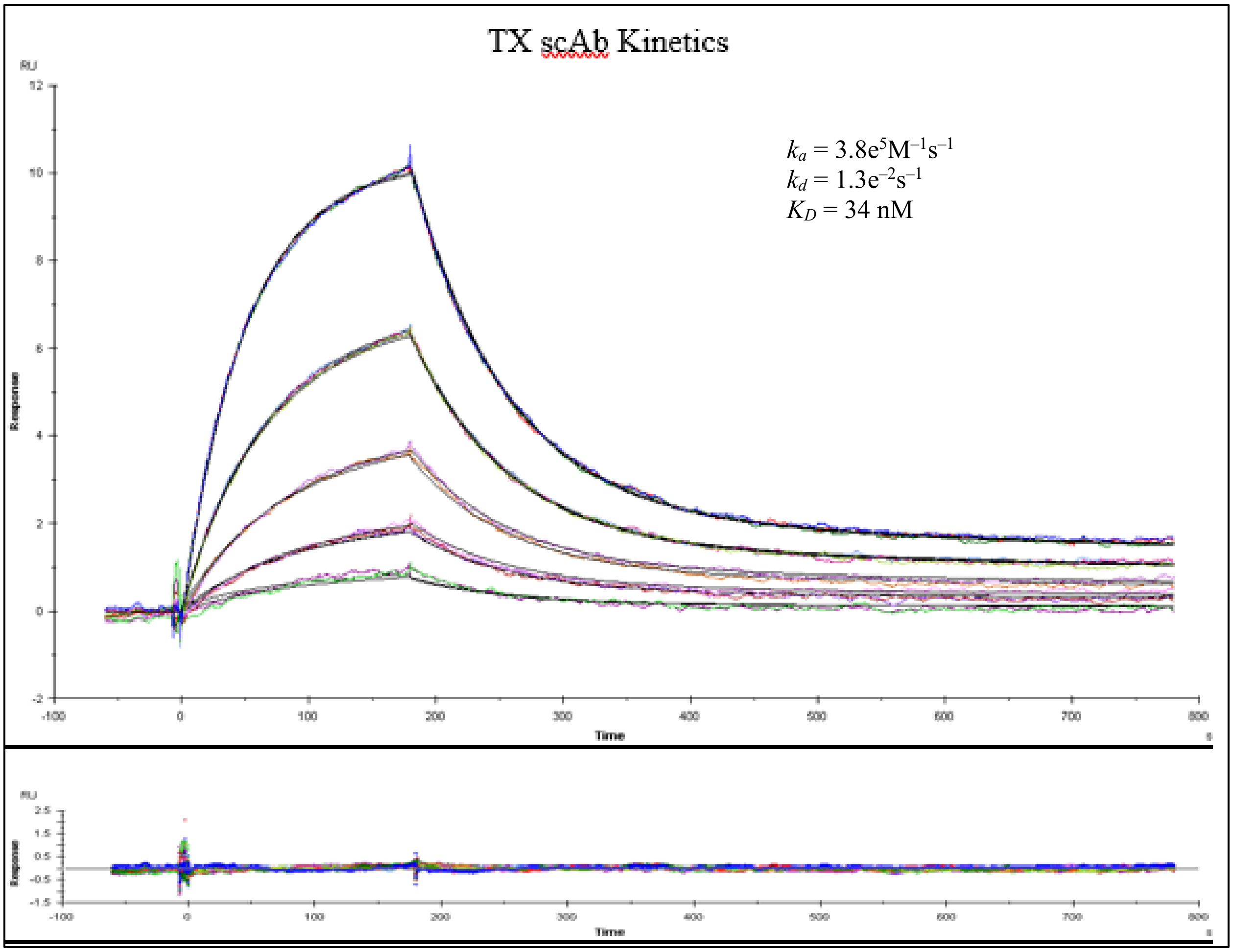


Figure 11. Kinetics of TXscAb determined using a Biacore T200 SPR system.

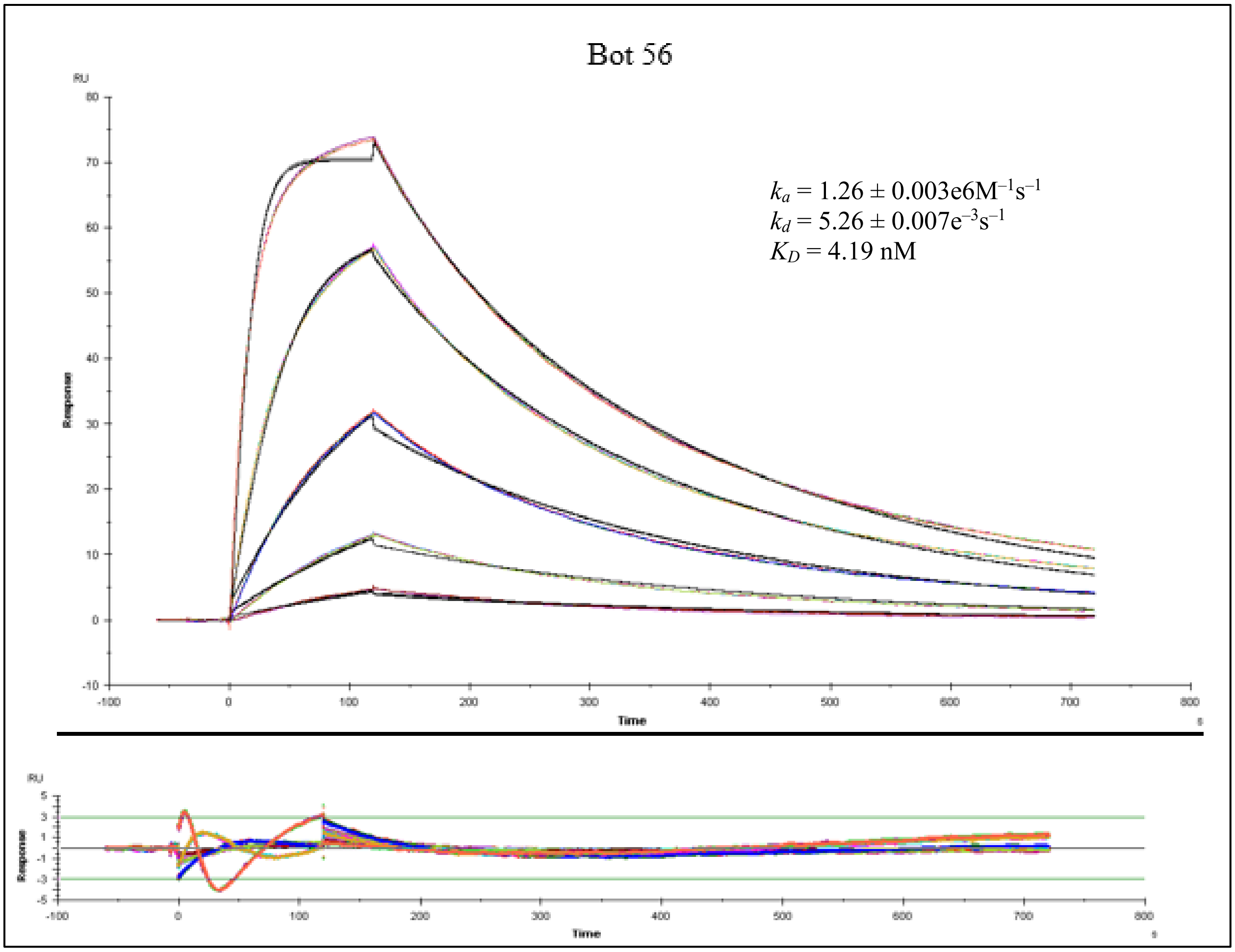


Figure 12. Kinetic fits with residuals of Bot 56, as determined using a Biacore T200 SPR system.

4. DISCUSSION

The standardized parametric tests established during the MS2 scFv antibody DARPA ATP (*3*) were used for this study, and molecular schemes for improving the thermal stability and affinity of an antibody for its target antigen were demonstrated. A snapshot of Bot 56 physical characteristics was obtained using the NanoDrop, Experion, and DLS measurement platforms, and these characteristics were compared with characteristics of the improved antibodies that were submitted by the University of Texas. TXscAb functional characteristics were measured using the DSC, ELISA, and SPR analytic platforms so that the effect of molecular engineering on thermal stability and affinity could be assessed.

An accurate assessment of protein concentration is critical for all of the test procedures described in this report. We applied the standard technique of spectrophotometry with the NanoDrop ND-1000 spectrophotometer. This allowed us to obtain the A_{280} of the sample, which is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with the A_{280} value to determine an accurate concentration.

After the concentration was determined, molecular weight and purity data were collected with the Experion automated electrophoresis system. This system employs microfluidic technology to automate electrophoresis for protein analysis. The results of the Experion analysis of the TXscAb protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software was typical for an scFv.

DLS was used in conjunction with the Experion electrophoresis system and NanoDrop ND-1000 spectrophotometer to evaluate how the protein behaved in solution. DLS data indicate the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data showed that TXscAb provided by the University of Texas was polydisperse: 99% of the sample's mass appeared to aggregate in solution (Figure 3). To mitigate the exacerbating effect of freeze–thawing on future sample aggregation, the antibody was aliquoted into single-use vials and centrifuged before use so that all testing would be consistent.

SPR was also used to obtain a kinetic analysis of the supercharged TXscAb binding to its target antigen HA33A, to compare binding parameters with the original antibody. The resultant K_D values clearly showed that TXscAb did not demonstrate the 100-fold improvement requested by DARPA.

The goal of this study was to modify a single protein to yield a 100-fold increase in the affinity and a 10 °C increase in the melting temperature of a provided protein. Bot 56 provided to each investigator had a 4.19 nM affinity for the recombinant protein HA33A and a melting temperature of 82.1 °C. The TXscAb sample provided by the University of Texas produced an affinity of 34 nM, which did not meet the 100-fold improvement requirement. TXscAb also had a melting temperature of 77.1 °C; although this did not improve the temperature, this protein did exhibit an excellent improvement in sustained activity at 75 °C.

5. CONCLUSION

The DARPA ATP sought to establish methods for rapidly engineering a given antibody reagent to exhibit physical and functional properties that far exceeded those of its native state, and to thereby expand user confidence in fielding antibody-based detection and diagnostic platforms in environments or operational scenarios that degrade or interfere with the currently available reagents. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP sought to obtain antibody reagents that could reliably function in harsh environmental conditions and increase the sensitivity of a sensor platform to detect lower levels of a threat agent.

This report documents the testing of an improved antibody produced by the University of Texas. This study evaluated the physical and functional characteristics of the antibody in the ECBC testing pipeline. Results were compared with the baseline characteristics of the original antibody's physical properties, to include concentration, molecular weight, purity, state of aggregation in solution, and functional measures such as binding affinity and thermal stability. The antibody supplied by the University of Texas exhibited enhanced thermal stability but no increase in affinity for binding to the HA33A protein antigen.

Blank

LITERATURE CITED

1. Frenzel, A.; Hust, M.; Schirrmann, T. Expression of Recombinant Antibodies. *Front. Immunol.* **2013,** *4*, 217.

2. Miklos, A.E.; Kluwe, C.; Der, B.S.; Pai, S.; Sicar, A.; Hughes, R.A.; Berrondo, M.; Xu, J.; Codrea, V.; Buckley, P.E.; Calm, A.M.; Welsh, H.S.; Warner, C.R.; Zacharko, M.A.; Carney, J.P.; Gray, J.J.; Georgiou, G.; Kuhlman, B.; Ellington, A.D. Structure-Based Design of Supercharged, Highly Thermoresistant Antibodies. *Chem. Biol.* **2012,** *19*, 449–455.

3. Buckley, P.E.; Calm, A.; Welsh, H.; Thompson, R.; Carney, J.; Warner, C.; Zacharko, M. *DARPA ATP Standardized Test Bed for Antibody Characterization: Characterization of an MS2 ScFv Antibody Produced by the University of Texas*; ECBC-TR-1434; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, May 2017; UNCLASSIFIED Report (AD1198330).

4. *Bio-Rad Experion Pro260 Analysis Kit Instruction Manual*, Rev C. Bio-Rad Laboratories: Hercules, CA, 2010, version 10000975, catalog no. 700-7101.

Blank

ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance at 280 nm
ATP	Antibody Technology Program
BGG	bovine γ-globulin
Bot	anti-botulinum toxin
CRP	Critical Reagents Program
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
HA33A	hemagglutinin-33/A
HPR	horseradish peroxidase
JPMO BSV	Joint Product Management Office for Biosurveillance
k_a	association rate constant
kan	kanamycin
k_d	dissociation rate constant
K_D	affinity constant
LB	Luria broth
MDB	milk diluent block
PBS	phosphate-buffered saline
R_{MAX}	maximum relative fluorescence unit
RT	room temperature
RU	response unit
scFv	single-chain variable fragment
SPR	surface plasmon resonance
strep	streptavidin
T_m	melting temperature
TXscAb	modified Bot 56 antibody produced by the University of Texas

Blank

DISTRIBUTION LIST

The following individuals and organizations were provided with one electronic version of this report:

U.S. Army Combat Capabilities Development
Command Chemical Biological Center
Biotechnology Branch
(DEVCOM CBC)
FCDD-CBR-BM
ATTN: Welsh, H.
Basi, K.

DEVCOM-CBC Technical Library
FCDD-CBR-L
ATTN: Foppiano, S.
Stein, J.

Defense Technical Information Center
ATTN: DTIC OA

U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER