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DARPA ANTIBODY TECHNOLOGY PROGRAM STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 SCFV ANTIBODY

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The selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay (ELISA), with little regard for quantification of the full spectrum of variables affecting antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV) Critical Reagents Program has recently instituted a quality program for standardization of test methods to support more comprehensive characterization and comparison of the physical and functional properties of antibody reagents within its repository. The development and standardization of antibody testing provides JPMO BSV with an invaluable platform for the provision of consistent, high-quality assays and reagents for current biodetection platforms as well as the development and validation of future systems. This platform will be used to characterize the MS2 recombinant antibody produced at U.S. Army Edgewood Chemical Biological Center for the Defense Advanced Research Projects Agency (DARPA) Antibody Technology Program (ATP).

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PREFACE

The work described in this report was supported by the Defense Advanced Research Projects Agency funding. The work was started in October 2010 and completed in September 2012.

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DARPA ANTIBODY TECHNOLOGY PROGRAM STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 SCFV ANTIBODY

1. INTRODUCTION

Current platforms for detection and diagnosis of biothreat agent exposure depend on the use of antibodies to recognize and bind to specific antigens. To date, the selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay (ELISA), with little regard for quantification of the full spectrum of variables affecting antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV) Critical Reagents Program recently instituted a quality program for the standardization of test methods to more fully characterize and compare the physical and functional properties of antibody reagents in its repository. The development and standardization of antibody testing provides the JPMO BSV with an invaluable platform for obtaining consistent, high-quality assays and reagents for existing biodetection platforms and also for the development and validation of future systems. This platform will be used to characterize the MS2 recombinant antibody produced at U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) for the Defense Advanced Research Projects Agency (DARPA) Antibody Technology Program (ATP).

The DARPA ATP focuses on developing technologies for enhancing the thermal stability and binding affinity of a given antibody. Functioning as an independent testing laboratory for this program, ECBC has provided specific technical support on immune reagents and defined the government-supplied antibody–antigen pairs. The goal of this project was twofold: (a) select, develop, and standardize the methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators; and (b) use those methods to validate the changes in antibody thermal stability and binding affinity that were achieved by the DARPA investigators. The antibody chosen for this project was the MS2 recombinant antibody produced at ECBC (*1*), which detects an MS2 coat protein (MS2CP) that forms the capsid for the MS2 bacteriophage. The focus of the work highlighted in this report was the evaluation of the original MS2 antibody that was supplied to DARPA-funded investigators for affinity and stability enhancement. The results of this study not only provide standardized parametric data on antibody properties and performance, but also contribute to the development of a decision analysis tool for expanding the confidence level during selection of antibody-based reagents that will optimize the field operational and performance metrics of future detection and diagnostic platforms.

2. MATERIALS AND METHODS

2.1 MS2 ScFv and MS2CP

MS2 single-chain variable fragment (scFv) antibody was produced from a plasmid supplied by Ellen Goldman (U.S. Naval Research Laboratory; Bethesda MD). The plasmid was designated Gv1, and the sequence was cloned into a pET22b(+) plasmid (EMD Millipore; Billerica, MA). Upon arrival in our laboratory, the plasmid was transformed into BL21 (DE3) *Escherichia coli* (EMD Millipore) by electroporation. Positive transformants were selected and grown to log phase in 1 mL cultures of terrific broth (TB; Life Technologies; Grand Island, NY) with 2% D-glucose (Sigma-Aldrich Company; St. Louis, MO) and 100 µg/mL of carbenicillin (Sigma-Aldrich) at 30 °C and were shaken at 250 rpm (Innova 4300 shaker; New Brunswick Scientific Company; Edison, NJ). Cultures were scaled-up to 50 mL using the same media formulation and grown overnight with the temperature reduced to 25 °C. In the morning, cells were harvested by centrifugation and resuspended in 500 mL of TB with carbenicillin and no glucose. Cells were then cultured for 3 h before being induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma-Aldrich). Cell paste was harvested by centrifugation 3 h after induction and flash-frozen in liquid nitrogen to aid in cell lysis. The pellet was weighed and resuspended in histidine-tag binding buffer composed of 20 mM sodium phosphate (pH 8.0), 0.5 M sodium chloride, and 20 mM imidazole (Sigma-Aldrich) at a 5:1 volume-to-weight ratio. The cell suspension was mechanically disrupted using an M-110p microfluidizer (Microfluidics; Westwood, MA) at 20,000 psi, and lysate was clarified via centrifugation. The clarified lysate was passed through a 0.45 µm filter before being applied to a 1 mL nickel affinity column (GE Healthcare Bio-Sciences Corporation; Piscataway, NJ). The protein was eluted in 20 mM sodium phosphate (pH 8.0), 0.5 M sodium chloride, and 0.5 M imidazole. Peak fractions were collected and separated on a 16/60 Superdex 200 gel filtration column (GE Healthcare Bio-Sciences). The fractions that corresponded with a monomeric protein were collected and flash-frozen in liquid nitrogen.

The MS2CP was produced from a pET-28a(+) plasmid (Novagen; Billerica, MA): the MS2CP sequence was inserted with an amino acid substitution of an arginine at position 83 in a construct engineered by DNA2.0 (Menlo Park, CA). Upon receipt, the lyophilized plasmid was rehydrated in ultrapure water and transformed into BL21 (DE3) SlyD-*E. coli* (Novagen) via electroporation. Positive transformants were selected and scaled up to a 100 L working volume in TB. The TB consisted of 12.0 g/L of casein peptone type T (Marcor Development Corporation; Carlstadt, NJ), 24.0 g/L of yeast extract (Marcor), 9.4 g/L of K_2HPO_4 (Fisher Scientific; Fair Lawn, NJ), 2.2 g/L of KH_2PO_4 (Sigma-Aldrich), and 0.4 mL/L of glycerol (Fisher Scientific; Pittsburgh, PA) with 0.075 mL/L of antifoam 204 (Sigma-Aldrich). The TB was autoclaved at 121 °C for 30 min. A sterile solution of kanamycin (Sigma-Aldrich) was used in all TB media at a working concentration of 40 mg/L.

A brief description of the scale-up procedure is as follows: One liter of seed cultures was prepared by inoculating 1 L of TB in a 4 L flask with 16 mL of a frozen stock of *E. coli* strain BL21 (DE3) SlyD-MS2CP-W83R and incubating in an Innova 4300 shaker at 30 °C and 220 rpm until the optical density (OD) at 600 nm (Genesys 20 spectrophotometer; Thermo Scientific; Waltham, MA) reached approximately 0.42. The seed cultures were then

used to inoculate an IF 150 fermentor (New Brunswick Scientific). A presterilized 2 L transfer bottle was used to aseptically transfer the cultures into the fermentor, which contained 100 L of presterilized TB. The controlled operating conditions for the fermentor included shaking at 400 rpm, airflow of 1 vvm (air volume per liquid volume per minute), temperature of 30 °C (pH 7.0, using sterile 3 M phosphoric acid or 3 M sodium hydroxide [Sigma-Aldrich] as necessary), and overhead pressure of 1 psi. When the OD inside the 100 L fermentor reached approximately 0.75, the cultures were induced with 100 mg/L of IPTG. Once the post-induction OD reached 4.8, the cells were harvested using a Carr Powerfuge continuous centrifuge (PneumaticScaleAngeles; Clearwater, FL) operating at 15,000 rpm with a flow rate of 500 mL/min. The cell paste was stored at –80 °C for later processing.

The cell paste was thawed and resuspended in phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich) at a 1:4 weight-to-volume ratio. The cell suspension was passed twice through an M-110Y microfluidizer (Microfluidics) at 20,000 psi and was spun in a J2-21M centrifuge (Beckman Coulter Life Sciences; Brea, CA) at 30,100 ×g for 2 h to remove debris. The crude lysate was passed through a 0.2 µm filter, mixed with Ni-NTA Superflow resin (Qiagen; Valencia, CA), and incubated overnight in an Innova 40 incubator shaker (New Brunswick Scientific) operating at 200 rpm and 4 °C.

The mixture of Ni-NTA resin and crude lysate was packed into a chromatography column using a BioCAD 700E system (Applied Biosystems; Foster City, CA) and was washed with 10 column volumes of 20 mM imidazole in PBS (pH 7.4). MS2CP was eluted with 300 mM imidazole in PBS (pH 7.4). Peak fractions were collected, and buffer was exchanged into PBS (pH 7.4) using a 470 mL packed volume of Sephadex G-25 fine gel chromatography media (GE Healthcare).

2.2 UV–Visible Spectrophotometry

A NanoDrop ND-1000 spectrophotometer (Thermo Scientific; Waltham, MA) was used to determine the MS2 scFv concentration and the absorbance of light at 280 nm (A_{280}) for the samples. The A_{280} value is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient is used in conjunction with the A_{280} value to determine an accurate concentration. The MS2 scFv concentration was determined by dividing the average A_{280} value by 1.77, which is the extinction coefficient for a scFv. This procedure was repeated for the MS2CP, except the extinction coefficient used to calculate concentration was 1.15, as determined from the amino acid sequence of the protein. Each reading required a 2 µL sample, which was placed on the sample pedestal. The arm of the instrument was lowered, creating a liquid column between the top of the arm and the surface of the pedestal; this was the path length through which the laser passed. The instrument was blanked using PBS, and readings were taken in triplicate. A positive control, bovine γ-globulin (BGG; Bio-Rad; Hercules, CA), was also tested to validate the instrument operation.

2.3 Electrophoresis

Molecular weight and purity data were collected with an Experion automated electrophoresis system (Bio-Rad). The system employs microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software, are designed to accomplish separation, staining, destaining, detection, and basic data analysis. The Experion Pro260 analysis kit uses engineered lower and upper internal alignment markers to provide clean baselines, accurate molecular weight sizing, and quantitative protein analysis (*2*). The Pro260 analytical software also determines sample purity by calculating the percent mass of the separated proteins in a sample. For Experion analysis, MS2 scFv was standardized to a final concentration of 1 mg/mL by diluting it in PBS and creating 20 mL aliquots for all testing. The BGG control and the sample (MS2 scFv) were then processed using a validated procedure included in the Bio-Rad Experion Pro260 analysis kit, rev. C (*3*). 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 priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip. The chip was then placed in the instrument, and the lid was closed, lowering the sample needles into the wells. The instrument was operated via the Experion software; each chip took 30 min to complete. All samples were run in triplicate alongside one sample of the BGG control and the Pro260 ladder. All analysis was performed using the Experion software.

2.4 Dynamic Light Scattering (DLS)

DLS was used to paint a picture of how the protein behaved in solution. DLS data indicates whether a protein is in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. Prediction algorithms within the software produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the MS2 scFv and the control bovine serum albumin (Sigma-Aldrich) were placed into a quartz 384-well 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 (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation. The plate was placed into a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Values were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using Dynamics software (Wyatt Technology). The results of the wells were averaged and reported.

2.5 Differential Scanning Calorimetry (DSC)

DSC was used to obtain a quantitative melting temperature (T_m) for the MS2 scFv protein. The T_m should predict the results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C predicts that the percent of antibody activity after the thermal stress test will remain above 50%. A T_m below 70 °C predicts at least 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 VP-DSC microcalorimeter (MicroCal; Northampton, 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 (in duplicate) were scanned from 15 to 100 °C at a rate of 60 °C/h. The transition midpoint of the protein was determined by data analysis using Origin 7.0 software (MicroCal).

2.6 Thermal Stress Test

All samples were diluted to 1 mg/mL before heat was applied to negate protective effects due to concentration (*2*). MS2 scFv was diluted to 1 mg/mL in 1× PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and was marked time 0. The remaining four aliquots were heated to 70 °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. These samples were then tested for activity.

2.7 ELISA

ELISAs were performed in triplicate using standard techniques. After the thermal stress test, each sample was diluted to 1 µg/mL in PBS and used to coat one row each of three Nunc MaxiSorp 96-well plates (Thermo Scientific), and 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 (Molecular Devices; Sunnyvale, CA). The plate was blocked with 1× milk diluent block (MDB) (KPL) for 30 min at 37 °C. The plate was washed, and PBS with 0.05% Tween 20 (PBS-T; Sigma-Aldrich) was applied to the plate such that each well received 100 µL. MS2CP was diluted in PBS-T to 2 µg/mL, and 100 µL was applied to the first well of each row. A twofold serial dilution was performed across the plate, and it was incubated for 1 h at 37 °C. After the plate was washed, mouse anti-MS2 (kindly supplied by the JPMO BSV Critical Reagents Program) was diluted to 5 µg/mL in 1× MDB, and 100 µL was added to each well. The plate was incubated at 37 °C for 1 h. The plate was washed, goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) (KPL) was diluted to 0.2 µg/mL in 1× MDB, and 100 µL was added to each well. The plate was incubated at 37 °C for 30 min. After the plate was washed, 100 µL of room-temperature 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) 1-component HRP substrate (KPL) was added to each well. After 20 min at 37 °C, the OD 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 software (GraphPad Software; La Jolla, CA).

2.8 SPR Methodology

One method for determining the kinetic parameters of antibody–antigen interactions is SPR. This is a rapid methodology for monitoring biomolecular interactions through excitation of surface plasmons. Polarized light is shone through a prism on a sensor chip with a thin metal film coating, which reflects the light by acting as a mirror. If the angle of light shone through the prism is changed, and the intensity of the reflected light is monitored, differences in intensity can be recorded. While the refractive index at the prism side of the chip is

not changing, the refractive index in the immediate vicinity of the metal surface will change when accumulated mass (bound proteins) adsorbs on the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this SPR angle shift provides information on the protein adsorption kinetics on the surface. The software can then provide an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions, as well as calculate the overall affinity constant (K_D) between antibody and antigen.

2.8.1 Thermostability Testing by SPR

Using a Biacore T200 system (GE Healthcare), 6500 response units (RUs) of MS2CP was tethered to one flow cell of a Biacore CM5 sensor chip using standard amine coupling chemistry. After a thermal stress test was performed, samples were centrifuged at 2000 ×*g* and 5 °C for 5 min. The analyte was run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated MS2 scFv sample at 400, 350, 300, 250, 200, 150, 100, and 50 nM and plotting the respective maximum analyte-binding capacity of the surface (R_{Max} value) in response units. Unheated and heated samples were then diluted 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 with an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Data was collected using the Biacore concentration analysis software, and the active concentration of heated sample was recorded. The running buffer used for this experiment was Biacore HBS-EP 1× buffer (GE Healthcare Life Sciences).

2.8.2 Kinetic Analysis by SPR

2.8.2.1 SPR Analysis Using a Biacore T200 System

Using a Biacore T200 system and HBS-EP 1× running buffer, 102 RU of MS2CP was tethered to a CM5 sensor chip using standard amine coupling chemistry. MS2 scFv 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 nM, 20 nM, 6.67 nM, 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 was analyzed using a Langmuir 1:1 fit.

2.8.2.2 SPR Analysis Using a Bio-Rad ProteOn XPR36 System

Using a ProteOn XPR36 SPR system (Bio-Rad) and PBS-T running buffer, 200 RU of MS2CP was tethered to a GLC sensor chip (Bio-Rad) using standard amine coupling chemistry. MS2 scFv was injected across the chip's surface for 120 s at a flow rate of 100 µL/min with a 600 s dissociation at 5 nM, 1.67 nM, 560 pM, 190 pM, and 60 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 100 µL/min. Data was analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Spectrophotometry Results

The MS2 scFv was read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings were as follows:

Replicate 1: 2.242
Replicate 2: 2.308
Replicate 3: 2.295

These three numbers were averaged and divided by the extinction coefficient of 1.77. The final concentration was determined to be 1.29 mg/mL.

3.2 Electrophoresis Results

The molecular weight of the MS2 scFv was determined using the Experion Pro260 analysis kit, as shown in Figure 1. The thick band in the middle of the second lane corresponds to the MS2 scFv antibody, and the fuzzy bands that surround it represent impurities. According to the Experion software, this sample was 94.1% pure and weighed 34.1 kDa.

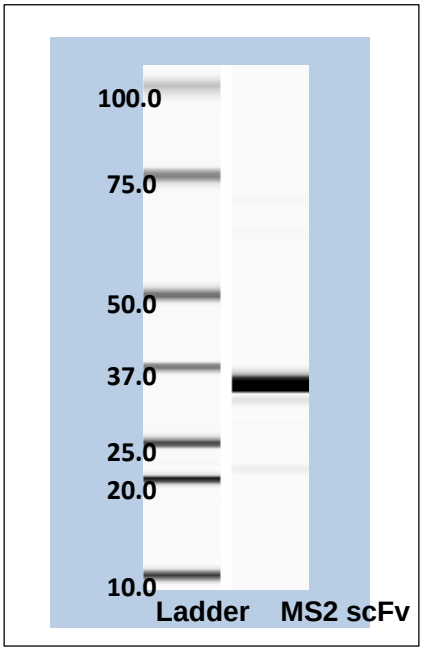

Figure 1. Molecular weight and purity. Digital gel of MS2 scFv produced by the Experion Pro260 analysis kit.

3.3 DLS Results

The MS2 scFv was analyzed in triplicate using the DynaPro plate reader. The radius of the MS2 scFv was determined to be 3.0 nm with a polydispersity of 13.3% (Table 1). Figure 2 contains representative correlation and regularization graphs for the MS2 scFV. The correlation graph (Figure 2A) depicts an asymptotic line indicative of a valid size distribution. The regularization graph (Figure 2B) illustrates the different forms of scFv that were identified in the sample. Table 1 shows the raw data produced for each replicate. Because 94% of the mass displayed favorable polydispersity and hydrodynamic radius, this sample preparation was considered to be monodisperse.

Table 1. Features of MS2 ScFv in Solution

Replicate	Radius (nm)	Polydispersity (%)
1	3.0	17.3
2	3.3	11.9
3	2.8	10.7
Average	3.0	13.3

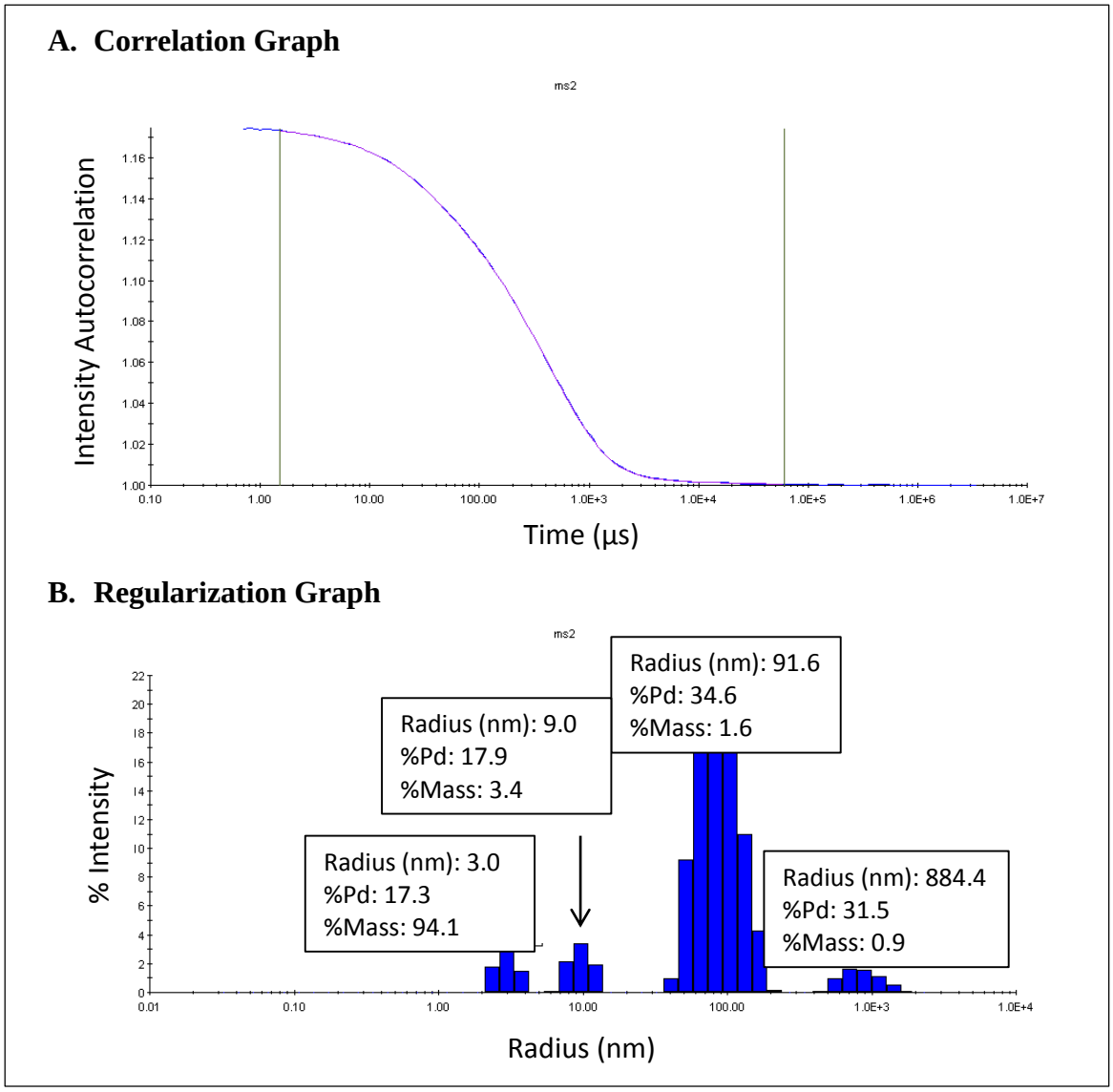

Figure 2. Radius and polydispersity (Pd) representation: (A) correlation graph and (B) regularization graph of the MS2 scFv used for determining the radius and polydispersity of the sample.

3.4 DSC Results

The MS2 scFv was read in duplicate on the MicroCal VP-DSC microcalorimeter. The peak T_m was 67.5 °C for both replicates. The final T_m was determined to be 67.5 °C, as shown in Figure 3.

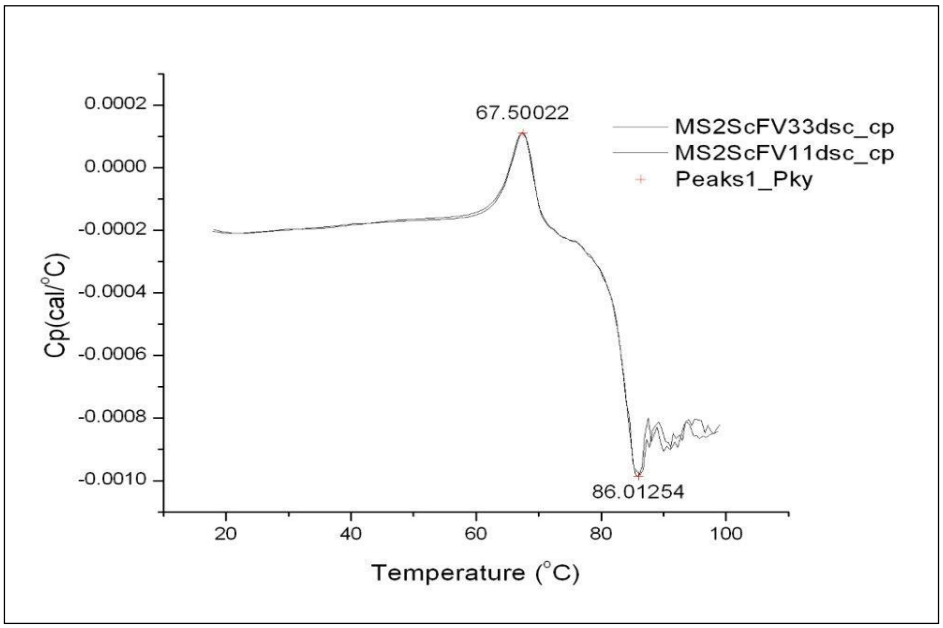

Figure 3. Transition midpoint. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the peak find function in Origin 7.0 software. The transition midpoint was calculated to be 67.5 °C.

3.5 ELISA Results

ELISAs were used to test the functional interaction of antibody and antigen after thermal stress at 70 °C. The ELISA data (Figure 4) show that when the MS2 scFv was heated to 70 °C, it lost all activity across all time periods of thermal stress. The curves in Figure 4A show antibody activity for different time points at 70 °C as a function of the concentration of antigen supplied. For each of the different time points, the area under the curve was calculated, averaged, and graphed (Figure 4B) to depict how the MS2 scFv reacted to thermal stress over time. This graph illustrates that less than 20% of the MS2 scFv remained functionally capable of binding to antigen after a 15 min exposure to 70 °C, and a near total loss of antigen binding occurred when MS2 scFv was heated for 60 min.

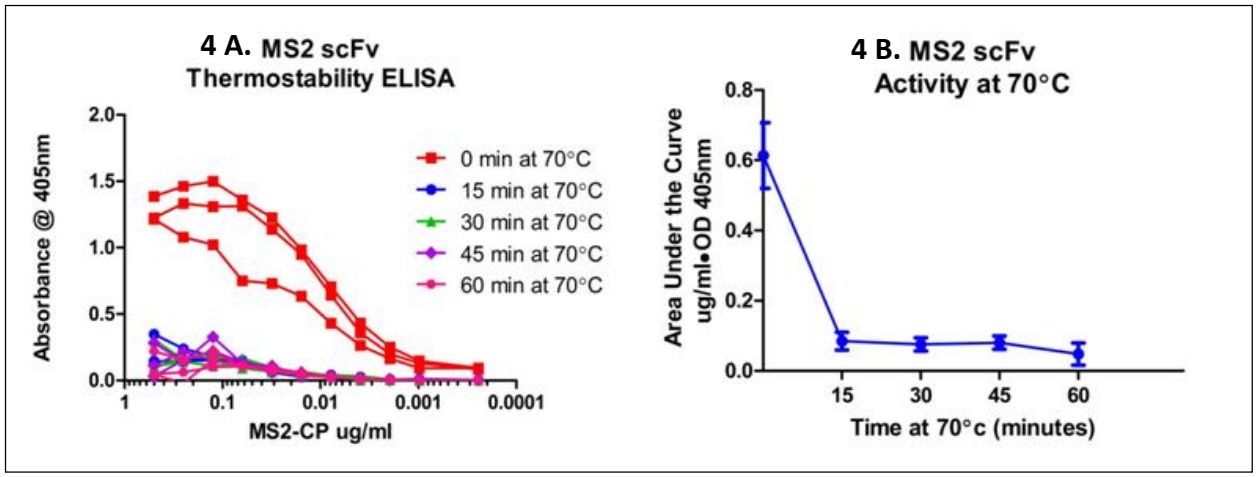

Figure 4. Thermostability of MS2 scFv by ELISA: (A) activity of MS2 scFv as a function of antigen concentration, and (B) area under the curve analysis depicting the effects of thermal stress.

3.6 SPR Results

3.6.1 Thermostability Testing by SPR

SPR was used to assess the functional binding between MS2 scFv and antigen after the antibody was heated to 70 °C for variable time periods. Five tubes of 1 mg/mL MS2 scFv were prepared and heated to 70 °C for 15, 30, 45, and 60 min time periods, which were followed by quenching on ice. A Biacore T200 system was used to compare the activity of each sample with a calibration curve for unheated sample. The percent activity of the heated samples was plotted over time (Figure 5). The results indicated that the scFv activity dropped off completely within the first 15 min at 70 °C.

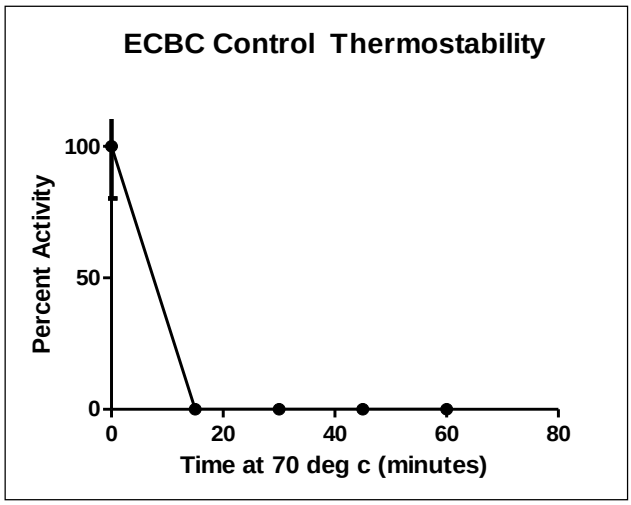

Figure 5. Thermostability of MS2 scFv was assessed using SPR. The antibody lost all ability to recognize the MS2CP target when heated for at least 15 min.

3.6.2 Kinetic Analysis by SPR

Kinetic analysis of the MS2 scFv binding to the MS2CP antigen was performed on two different SPR-based instruments. Results from a direct binding SPR experiment using the Biacore T200 system are presented in Figure 6A. Data was normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using Biacore T200 system software. The K_D was determined to be 15.5 nM. Results from experiments run on the ProteOn XPR36 system are presented in Figure 6B. For these experiments, the MS2CP antigen was covalently coupled across five channels of a ProteOn GLC chip, leaving one reference channel blank. Using a one-shot kinetics injection, five concentrations of MS2 scFv were injected across the protein surface. Data was processed using ScrubberPro software (Biosensor Tools; Salt Lake City, UT). Using the ProteOn XPR36 system, the K_D value was determined to be 8.66 nM when the data was fitted to a Langmuir 1:1 model.

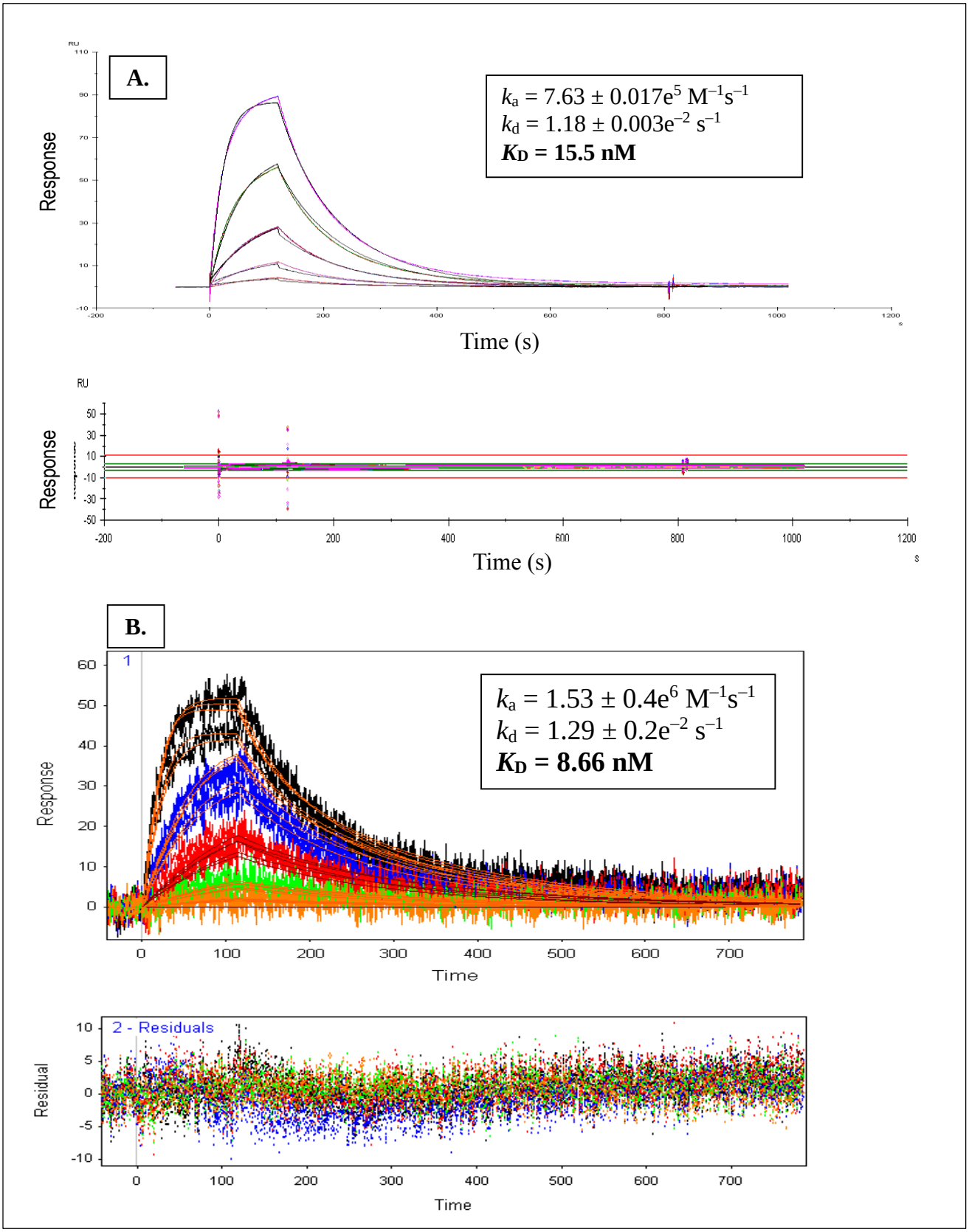

Figure 6. Kinetic fits with residuals of the MS2 scFv, as determined using (A) a Biacore T200 system, and (B) a ProteOn XPR36 system.

4. DISCUSSION

This study established and standardized the parametric tests for performance on the MS2 scFv antibody. This antibody was selected by the DARPA ATP as the initial substrate for performers to use in demonstrating their molecular schemes for improving the thermal stability and affinity of an antibody for its target antigen. The test bed developed was used to define the physical and functional properties of the reference MS2 scFv antibody and establish the baseline for subsequent testing of the engineered antibodies submitted by the ATP performers. A snapshot of the MS2 scFv's physical characteristics was obtained using the NanoDrop, Experion, and DLS measurement platforms. Measurements of the MS2 scFv's functional characteristics, for assessing the effects of molecular engineering on thermal stability and affinity, were obtained using the DSC, ELISA, and SPR analytic platforms.

An accurate assessment of protein concentration is critically important for all of the test procedures described in this report. We applied the standard technique of spectrophotometry with the NanoDrop ND-1000 system. This instrument was employed to provide the A_{280} value 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 A_{280} value to determine an accurate concentration.

After concentration was determined via spectrophotometry with the NanoDrop system, 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 Experion analysis of the MS2 scFv protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software (shown in Figure 1) was typical for an scFv.

DLS was used in conjunction with the Experion and NanoDrop systems to illustrate how the protein behaved in solution. DLS data indicates 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 established whether the MS2 scFv provided to the ATP performers was monomeric and monodisperse. Only 6% of the sample mass appeared to be aggregating in solution (Figure 2). To mitigate the exacerbating effects of freeze-thawing on future sample aggregation, the MS2 scFv was aliquoted into single-use vials and centrifuged before use, so that all future testing would be consistent.

In the next round of testing, the thermostability of MS2 scFv was evaluated using DSC, ELISA, and SPR. DSC was utilized to identify a quantitative T_m for MS2 scFv. The T_m should predict the results of ELISA and SPR thermostability testing. A T_m above 70 °C predicts that the percent activity for MS2 scFv after thermal stress will remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in MS2 scFv activity after thermal stress. The MS2 scFv antibody described herein exhibited a T_m of 67.5 °C (Figure 3); therefore, it was expected that heating this sample above 67.5 °C would cause the antibody to unfold and lose at least 50% of its activity as evaluated by ELISA and SPR.

The results of the thermal stress test demonstrated that the MS2 scFv was almost totally inactivated within the first 15 min of heating at 70 °C and at each additional time point up to the 1 h test limit. Both the ELISA and SPR data confirmed that the MS2 scFv reference antibody was unable to bind the MS2CP after only 15 min of heating at 70 °C (Figures 4 and 5).

SPR was also used to obtain a kinetic analysis of MS2 scFv binding to its target antigen (MS2CP), to serve as a baseline for comparing binding parameters between the original antibody and those antibodies engineered for better performance by the DARPA ATP performers. Kinetic data for MS2 scFv binding to MS2CP was obtained using the Biacore T200 and ProteOn XPR36 SPR platforms. The Biacore T200 platform yielded a K_D of 15.5 nM, whereas the ProteOn XPR36 platform yielded a K_D of 8.66 nM (Figure 6). These two numbers are statistically the same, within the error of SPR measurements.

5. CONCLUSION

The DARPA ATP seeks to establish methods for rapidly engineering a given antibody reagent to exhibit physical and functional properties that far exceed those of its native state, thereby expanding user confidence in fielding antibody-based detection and diagnostic platforms in environments or operational scenarios in which currently available reagents exhibit degradation or interference. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP will develop antibody reagents that can reliably function in harsh environmental conditions and increase the sensitivity of sensor platforms to detect lower levels of threat agents.

This report documents the development of a standardized test bed for the physical and functional characterization of native and engineered antibodies. We expressed and purified a recombinant MS2 scFv and evaluated its physical and functional characteristics in our testing pipeline. The test bed was developed to provide baseline characteristics of a given antibody's physical properties, such as concentration, molecular weight, purity, and state of aggregation in solution, as well as functional measures, such as binding affinity and thermal stability. The test bed and work flow established herein will be used to characterize and assess whether MS2 scFv antibody variants produced under the DARPA ATP exhibit enhanced thermal stability or affinity for binding to the MS2CP antigen.

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ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance of light at 280 nm
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ATP	Antibody Technology Program
BGG	bovine γ-globulin
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
HRP	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactoside
JPMO BSV	Joint Product Management Office for Biosurveillance
k_a	association rate constant
k_d	dissociation rate constant
K_D	affinity constant
MDB	milk diluent block
MS2CP	MS2 coat protein
OD	optical density
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20
Pd	polydispersity
R_{Max}	maximum analyte-binding capacity of a surface
RU	response unit
scFv	single-chain variable fragment
SPR	surface plasmon resonance
TB	terrific broth
T_m	melting temperature

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