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Evaluation of Recombinant Antibodies Produced in the Expi293 and ExpiCHO (Thermo) Expression Systems for Use in Immunoassays

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EXECUTIVE SUMMARY

The objective of this study is to transition antibodies, developed as part of the Defense Advanced Research Projects Agency (Arlington, VA) Antibody Technology Program (ATP), to produce analogous thermostable recombinant antibodies and immunoassays that are of interest to the Defense Biological Product Assurance Office (DBPAO; Fort Detrick, MD). The development of an immunoassay with thermostable antibodies may reduce or eliminate the need for cold-chain logistics in the transportation and deployment of biological sensors. Two antibodies from ATP, recombinant α -MS2 immunoglobulin G (IgG) (Rec α -MS2) and recombinant α -hemagglutinin 33 (HA33) IgG (Rec α -HA33), were reproduced by members of the U.S. Army Edgewood Chemical Biological Center (ECBC; now known as the U.S. Army Combat Capabilities Development Command Chemical Biological Center; Aberdeen Proving Ground, MD) using transient transfections in mammalian cell lines. The products were assessed for their thermostability and utility in immunoassays. Rec α -MS2 was assessed for its activity against the recombinant coat protein of bacteriophage MS2, a nonenveloped ribonucleic acid virus commonly used as a simulant for biological threat viruses. Rec α -HA33 was assessed for its activity against recombinant botulinum neurotoxin HA33, a surface protein of botulinum neurotoxin, which is one of the most toxic substances known to mankind. Our results demonstrate high reproducibility of the recombinant antibodies with appropriate activities. Compared with other DBPAO antibodies, Rec α -MS2 did not perform well when used to develop an immunoassay, especially for binding intact MS2 bacteriophage. Differences in the three-dimensional structure between intact MS2 bacteriophage and the recombinant MS2 CP, which was used as a biopanning target of the original α -MS2 antibody, may be responsible for the lack of binding to intact MS2. In contrast, Rec α -HA33 was successfully used to develop a sandwich-type immunoassay for both recombinant HA33 and botulinum neurotoxin A toxoid. These results demonstrate the utility of the ATP antibodies together with the ability of ECBC members to produce recombinant antibodies by various methods and to develop novel immunoassays for biological threat agents.

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PREFACE

The work described in this report was started in January 2015 and completed in August 2017.

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EVALUATION OF RECOMBINANT ANTIBODIES PRODUCED IN THE EXP1293 AND EXPICHO (THERMO) EXPRESSION SYSTEMS FOR USE IN IMMUNOASSAYS

1. INTRODUCTION

The capability of antibodies to recognize antigens with specificity makes them valuable tools for biological detection systems. Many antibody-based sensor assays are used for rapid biological threat detection; however, many of these assay systems have functional limitations in the field because the reagents must be stored at low temperatures. Cold-chain logistics in transport and deployment are required to maintain the optimal performance of most antibody-based assays. Therefore, the amounts and varieties of antibody-based assays that are kept in laboratories and stockpiled are limited because of competition for refrigerated storage space. The Defense Advanced Research Projects Agency (Arlington, VA) launched the Antibody Technology Program (ATP) in 2009 to generate high-affinity, highly stable antibodies through the design of stable antibody structure frameworks. Specificity was achieved with grafting and maturation through in vitro somatic hypermutation.¹ In this study, two thermostable antibodies from ATP, recombinant α -MS2 immunoglobulin G (IgG) (Rec α -MS2) and recombinant α -hemagglutinin 33 (HA33) IgG (Rec α -HA33), were reproducibly manufactured and used for antibody-based detection assays.

Rec α -MS2 was assessed for its activity against the recombinant coat protein (CP) of bacteriophage MS2, a nonenveloped ribonucleic acid (RNA) virus commonly used as a simulant for biothreat viruses. The MS2 virion contains an icosahedral capsid, 178 copies of CP, a single A-protein (AP), and a positive-sense, single-strand RNA genome. The virus is about 28 nm in diameter with a genome of only 3569 nucleotides, and the CP is composed of 130 amino acids.² MS2 phage is neither toxic nor harmful to animals; moreover, it can be produced on a large scale using industrial fermentation systems. Thus, MS2 is an ideal simulant for pathogenic viruses and has been used as such to evaluate systems for the detection of variola and hemorrhagic fever viruses.³

Rec α -HA33 was assessed for its activity against recombinant botulinum neurotoxin HA33, a surface protein of botulinum neurotoxin, which is one of the most toxic substances known. HA33 is a 33 kDa subcomponent of neurotoxin-associated proteins produced by botulinum neurotoxin A, which binds to the molecules and other proteins of the host cell surface. There are six HA33 proteins in each complex that are resistant to protease digestion, a feature likely to be involved in the protection of the botulinum neurotoxin from proteolysis.⁴ The transient transfections of Rec α -HA33 and Rec α -MS2 were performed in mammalian cell lines to generate large-scale productions of each recombinant antibody. Once binding activities and thermostability were validated, both antibodies were used to develop bead-based sandwich immunoassays on the MAGPIX instrument (Luminex Corporation; Austin, TX) to assess their function and usability for immunoassays.

2. METHODS

2.1 Antibody Production

Recombinant antibody production was performed by transfecting mammalian cells with antibody plasmid expression constructs. Rec α -MS2 was produced using two plasmids in accordance with the AnaptysBio method (San Diego, CA).⁵ One coding was used for the light chain (LC), and the other was used for the heavy chain (HC) and co-transfected in Expi293F (Thermo Fisher Scientific; Wilmington, DE) suspension cells. All reagents and protocols were from the Life Technologies ExpiFectamine 293 (Thermo Fisher) transfection kit. Transfections were optimized in 24-well plates before they were scaled up to large-volume transfections. The cells were diluted to 2.5×10^6 /mL of Expi293 medium before each transfection. The ratio of LC/HC deoxyribonucleic acid (DNA) was varied from 1:1 to 35:1 in 1 mL cultures (1 μ g of total DNA/1 mL transfection volume). The cells were incubated at 37 °C with 8% CO₂, at 90% relative humidity and 195 rpm. Scale-up productions slowed the shaking speed in accordance with the vessel size. After eight days of post-transfection, supernatants were subjected to protein A affinity purification by 1 mL protein A HP SpinTrap columns (GE Healthcare Life Sciences; Pittsburgh, PA) for small volume optimizations, or by AKTA Protein A purification (GE Healthcare) for large-scale productions.

The Rec α -HA33 sequence was coded into one plasmid in which the HC and LC were synthesized in a bi-cistronic vector with an internal ribosomal entry site by ATUM (Menlo Park, CA) and transfected in ExpiCHO-S suspension cells (Thermo Fisher). Transfection conditions were optimized in 30 mL volumes using the standard protocol from the Expifectamine CHO expression system transfection kit (Thermo Fisher) before scaling up to larger-volume transfections. When the cells were cultured in 280 mL transfections, the MAX titer protocol was used, and the speed of the shaker was decreased to 110 rpm. The other cell culture conditions remained the same. After transfection, sample supernatants were collected at 13 days post-transfection and subjected to protein A affinity purification using the AKTA system.

2.2 Validation of IgG Activity and Thermostability

To validate IgG activity, an indirect enzyme-linked immunosorbent assay (ELISA) and a kinetic analysis using biolayer interferometry (BLI) were performed with purified antibodies. For the ELISA, 96-well assay microplates (Nunc; Rochester, NY) were coated with either 2.0 μ g/mL of recombinant MS2 CP or recombinant HA33 (both obtained from the U.S. Army Edgewood Chemical Biological Center [ECBC], now known as the U.S. Army Combat Capabilities Development Command Chemical Biological Center [DEVCOM CBC]; Aberdeen Proving Ground, MD) in phosphate-buffered saline (PBS) at 4 °C overnight. After being washed with wash solution (KPL Inc.; Gaithersburg, MD) and blocked with KPL milk diluent/blocking solution for 30 min, each antibody was added to 20 μ g/mL blocking solution with 1:2 serial dilutions for 1 h of incubation. After washing, KPL peroxidase-labeled goat anti-human antibody (diluted in blocking solution) was added for 1 h of incubation. The plates were then washed and

developed using KPL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid substrate. The plates were read at 410 nm on a Biotek Instruments (Winooski, VT) plate reader, and the data were analyzed using BioTek Gen 5 2.00 software and Microsoft Excel.

BLI was used to measure and analyze antibody binding activity. The Octet RED384 System (Pall ForteBio; Fremont, CA) was used to measure binding activity for both recombinant antibodies. Amine reactive second-generation (AR2G) biosensors (Pall ForteBio) were first soaked in water for 10 min and then in kinetics buffer (Pall ForteBio) for 1 min to determine a baseline signal. Biosensors were activated in 10 nM *N*-hydroxysuccinimide (NHS) and 20 nM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride buffer for 10 min. Then, 25 µg/mL of respective antigen diluted in sodium acetate at pH 5.0 was directly immobilized onto the sensors for 5 min. After quenching with 1 M ethanolamine for 2 min, the sensors were associated with a titration series for each antibody for 5 min to capture the full binding analysis.

The thermostability of each recombinant antibody was assessed in the same manner. Both antibodies were first divided into multiple samples, which were subjected to incubations at variable temperatures for variable time periods. With one incubation temperature and time duration per sample, the thermostability of each antibody could be assessed in a time- and temperature-dependent manner. The binding activity of heated samples was compared with that of nonheated reference samples. Binding activity was measured and analyzed using the Octet RED384 System.

2.3 Luminex MAGPIX Immunoassay Development

Capture sandwich assays were performed and analyzed using the MAGPIX platform. Each antibody was immobilized on Luminex MagPlex carboxylated microspheres at 5 pg antibody/microsphere using the carbodiimide coupling protocol provided by the manufacturer. To conduct the coupling, 0.1 M of sodium phosphate monobasic (pH 6.2 ± 0.2) was used for washing the beads and incubation. The microspheres were then washed and resuspended in 0.01 M PBS (pH 7.4 ± 0.1) with 0.05% Tween 20. Antibodies immobilized on microspheres were used as the capture medium in the assay. Detection antibodies were biotinylated using a 30-fold molar excess of succinimidyl-6-[biotinamido]-6-hexanamido hexanoate (NHS-LC-LC-Biotin) (Thermo Scientific, Rockford, IL) as the detection element, and a streptavidin-phycoerythrin conjugate (SAPE) served as the tracer molecule. Antibody-coated microspheres (2500 per well) were incubated with serial dilutions of samples diluted in PBS + 0.5% Triton X-100. The reactions were incubated for 1 h, washed, and then incubated with 4 µg/mL of biotinylated antibody for 30 min. The microspheres were washed again and then incubated with 4 µg/mL of SAPE to generate the fluorescent complex. After a final wash and resuspension in 100 µL of wash buffer, the assay results were evaluated using the MAGPIX platform. Incubations were performed at room temperature at 800 rpm and protected from light. Samples were washed twice with 100 µL of wash buffer (0.01 M PBS with 1% of bovine serum albumin and 0.05% sodium azide at pH 7.4) and then analyzed on the MAGPIX instrument.

3. RESULTS

3.1 Production of Recombinant Antibodies

Large amounts of recombinant antibodies were generated using the transient transfections. Optimization transfections of Rec α -MS2 showed that 1 μ g/mL of DNA and a 4:1 ratio of LC/HC produced the highest protein yield compared with other ratios or quantities of DNA (data not shown). A 1 L volume of Expi293 cells transfected with 1 μ g/mL of DNA produced 52.2 mg of Rec α -MS2. Optimization transfections of Rec α -HA33 showed that transfection with 0.5 μ g/mL of DNA resulted in the highest recombinant protein concentration (data not shown). In contrast to Rec α -MS2 production in Expi293 cells, a 280 mL volume of ExpiCHO cells transfected with 1 μ g/mL of DNA produced 110.5 mg of Rec α -HA33. Affinity purified recombinant antibodies were analyzed for purity using the Experion lab-on-a-chip Pro260 instrument. Figure 1 shows that the purity of each antibody was >95%.

IgG antibodies are composed of two polypeptide chains, HC and LC. Figure 1 shows that the HC was approximately 50 kDa, and the LC was approximately 25 kDa. The ladder on the left side of the figure is the molecular weight standard.

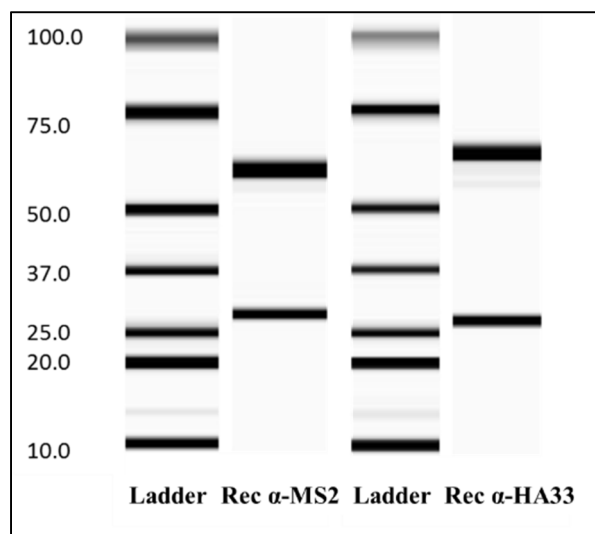


Figure 1. Purity analysis of Rec α -MS2 and Rec α -HA33. (Left) purity of Rec α -MS2 is shown at 98.25% and (right) purity of Rec α -HA33 is shown at 95.4%.

3.2 Validation of Rec α -MS2 and Rec α -HA33 Binding and Thermostability

Validation of Rec α -MS2 and Rec α -HA33 binding and thermostability was performed by using recombinant proteins in indirect ELISA and BLI platforms. BLI is an optical analytical technique for measuring biomolecular interactions and analyzing 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 binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift. These interactions are measured in real time,

and they can monitor binding specificity, rates of association and dissociation, and concentration.⁶ The results are shown in Figure 2 and Table 1. Both recombinant antibodies have strong affinities to their respective recombinant proteins. Figure 3 shows that each antibody maintains binding affinity to its respective recombinant protein after heating at 70 °C for 1 h. However, after heating at 75 °C, binding affinity for both antibodies is weakened. These results clearly demonstrate the thermostability of these recombinant α -MS2 and α -HA33 antibodies, whereas previous work revealed that most IgGs lost more than 75% activity at 75 °C.*

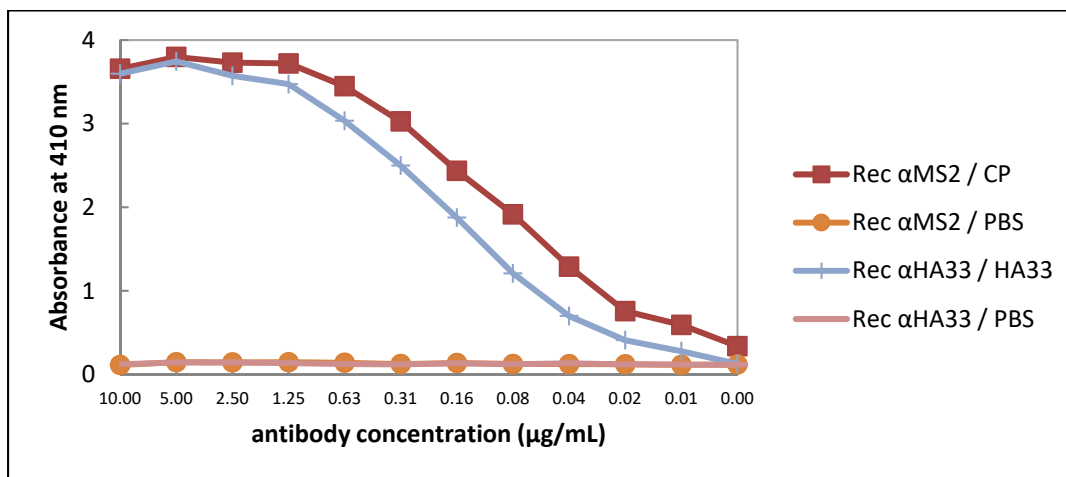


Figure 2. Indirect ELISA data of Rec α -MS2 and Rec α -HA33.

The vertical axis in Figure 2 shows absorbance measured at 410 nm in respective wells coated by recombinant MS2 CP, recombinant HA33, and PBS. A 1:2 titration series of the antibodies, beginning with 10 μ g/mL and ending with 9.77 ng/mL, was used to confirm that each antibody had specific binding to the respective antigen.

Table 1. BLI Kinetic Analysis Data of Rec α -MS2 and Rec α -HA33

	Rec α -MS2	Rec α -HA33
K_D	54.6 nM	1.1 nM
K_a	1.57E+05	1.18E+04
K_d	9.17E-07	3.55E-04

K_D, the equilibrium dissociation constant.

K_a, the binding constant.

K_d, the dissociation constant.

Kinetic analysis was conducted using BLI. The target antigen, recombinant MS2 CP or recombinant HA33, was directly immobilized onto AR2G sensors. A 1:2 titration series of the antibodies, beginning with 125 μ g/mL and ending with 1.95 μ g/mL, was used to capture the full binding analysis.

*Patricia E. Buckley, U.S. Army Edgewood Chemical Biological Center; Aberdeen Proving Ground, MD, personal communication, 25 July 2017.

The vertical axis in Figure 3 shows ligand-binding activity of heated antibodies, which was measured using BLI. Each sample was heated at 70 or 75 °C for the indicated time up to 1 h to determine the antibody thermostability.

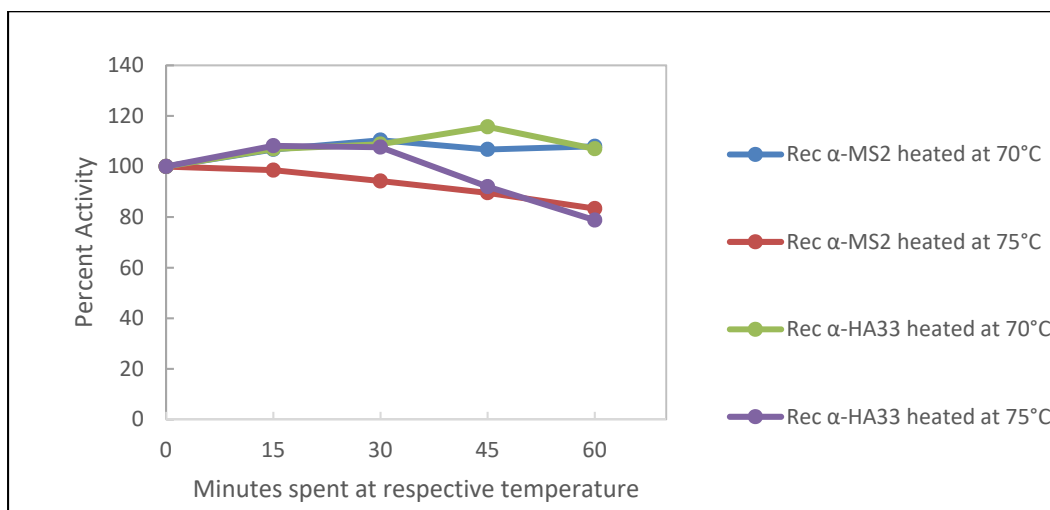


Figure 3. Percent activity of heated antibodies.

3.3 Rec α -MS2 and Rec α -HA33 Immunoassay Development and Analysis

Sandwich-type immunoassays were developed using Rec α -MS2 and Rec α -HA33 for analysis with the MAGPIX detection system. The function of each antibody was assessed by comparing results using analogous polyclonal antibodies provided by the Defense Biological Product Assurance Office (DBPAO; Fort Detrick, MD): Rabbit anti-MS2 (Rab α -MS2: ABE#120, J-291100-02) and rabbit anti-botulinum A antibody (Rab α -BOT: ABE#112, AB-R-BOT-A J-301000-03). Figures 4 and 5 show that the Rab α -MS2 had greater sensitivity than Rec α -MS2 in the MAGPIX immunoassays for detecting recombinant MS2 CP and intact MS2 phage. Most notably, Rec α -MS2 had considerably weaker binding to intact MS2 when compared with Rab α -MS2. When α -MS2 was generated by ATP, the single-chain variable fragment was derived from an ECBC library generated from mice immunized with MS2 phage.⁴ The original antibody was isolated using the recombinant CP, and it showed greater sensitivity than intact MS2 phage. The recombinant antibody may have reflected sensitivity for the recombinant protein because it was the original isolating target. However, this may also indicate that the epitope recognized and bound by Rec α -MS2 was partly obscured when the CP was in the three-dimensional structure of the intact viral coat.⁶ The α -MS2 epitope specificity was conserved from the original antibody to the recombinant antibody produced in this study.

Signal intensity of sandwich-type MAGPIX immunoassay for recombinant MS2 CP was compared with four antibody pairs using Rec α -MS2 or Rab α -MS2 (Figure 4). Each legend pairing shows an antibody coupled with microsphere (capture antibody)/biotinylated antibody (detection antibody), respectively. The vertical axis shows the mean fluorescence intensity divided by the mean fluorescence intensity of an antigen-free sample represented as the signal to noise (S/N) ratio. The data in this figure are based upon two replicates generated at each test concentration.

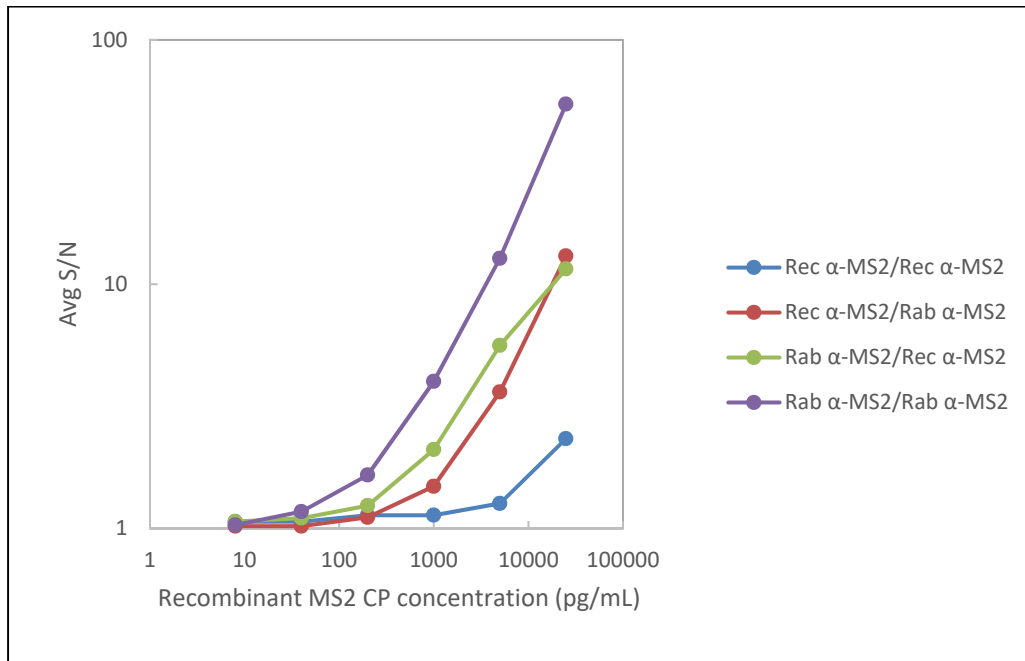


Figure 4. Luminex MAGPIX immunoassay result of recombinant MS2 CP.

Signal intensity of the sandwich-type MAGPIX immunoassay for intact MS2 bacteriophage (Delft Technical University; Delft, South Holland) was compared with four antibody combinations using Rec α -MS2 or Rab α -MS2 (Figure 5). Each legend pairing shows an antibody coupled with microsphere (capture antibody)/biotinylated antibody (detection antibody), respectively. The vertical axis shows the mean fluorescence intensity divided by the mean fluorescence intensity of antigen-free sample, represented as an S/N ratio. The data in this figure are based upon two replicates generated at each test concentration.

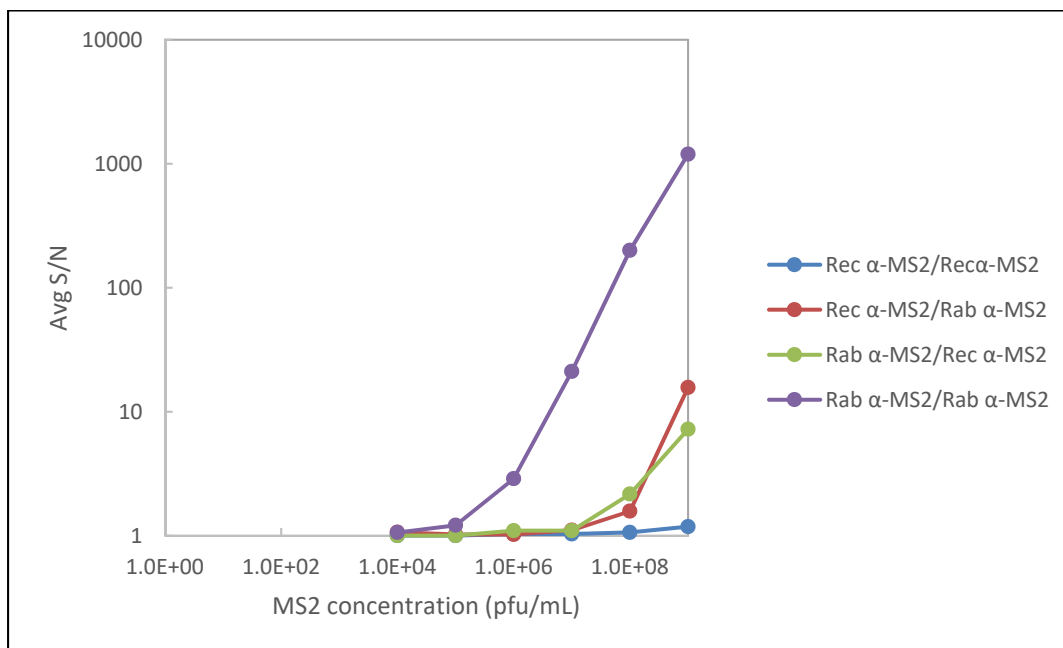


Figure 5. Luminex MAGPIX immunoassay result of intact MS2.

Figures 6 and 7 show that Rec α -HA33 had greater sensitivity in detecting recombinant HA33 and botulinum A toxoid (procured from ECBC), in particular when the recombinant antibody was used as the capture antibody. Although the difference in signal strength between Rab α -BOT and Rec α -HA33 as the capture antibody was minimal, the results indicate that Rec α -HA33 generally performed better than the polyclonal antibody. In contrast, the Rab α -BOT functioned better as the detector. As a polyclonal antibody, it recognized more epitopes on the toxoid captured by the α -HA33 antibody coupled microspheres. Lastly, to further assess Rec α -HA33, the limit of detection (LOD) was refined using Rec α -HA33 as the capture antibody and Rab α -BOT as the detector antibody. In these MAGPIX immunoassays, LOD was determined as the concentration of recombinant protein or toxoid that produced an S/N over 2.0. Tables 2 and 3 show that the LOD was between 5 and 10 pg/mL when the target was recombinant HA33 and between 200 and 400 ng/mL when the target was botulinum A toxoid. Unsurprisingly, the antibody pair was less able to bind to the toxoid than to the purified recombinant protein. The toxoid was chemically inactivated by cross-linking, which also eliminates epitopes.

Signal intensity of sandwich-type MAGPIX immunoassay for recombinant HA33 was compared with four antibody combinations using Rec α -HA33 or Rab α -BOT (Figure 6). Each legend pairing shows an antibody coupled with microsphere (capture antibody)/biotinylated antibody (detection antibody), respectively. The vertical axis shows the mean fluorescence intensity divided by the mean fluorescence intensity of an antigen-free sample, represented as an S/N ratio. The data in this figure are based upon two replicates generated at each test concentration.

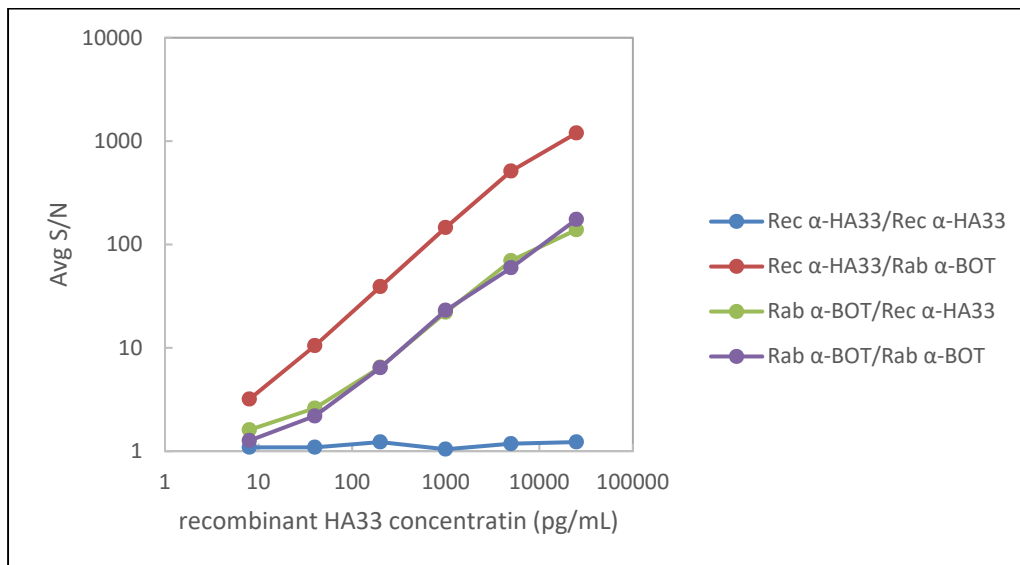


Figure 6. Luminex MAGPIX immunoassay result of recombinant HA33.

Signal intensity of sandwich-type MAGPIX immunoassay for botulinum A toxoid was compared with four antibody combinations using Rec α -HA33 or Rab α -BOT (Figure 7). Each legend pairing shows an antibody coupled with microsphere (capture antibody)/biotinylated antibody (detection antibody), respectively. The vertical axis shows the mean fluorescence intensity divided by the mean fluorescence intensity of antigen-free sample, represented as an S/N ratio. The data in this figure are based upon two replicates generated at each test concentration.

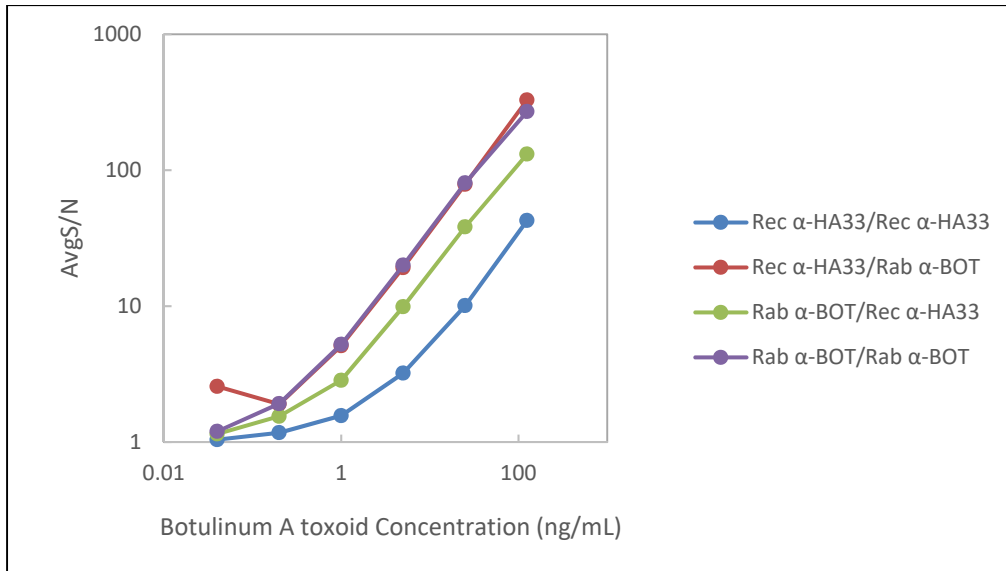


Figure 7. Luminex MAGPIX immunoassay result of botulinum A toxoid.

The vertical axis in Figure 8 shows the mean fluorescence intensity (MFI) at each sample concentration. To refine the LOD of each respective antigen, four separate runs of 24 replicates at each concentration of antigen were performed on two different MAGPIX instruments by two different operators on two separate days. The data in this figure are based upon 16 replicates generated at each test concentration.

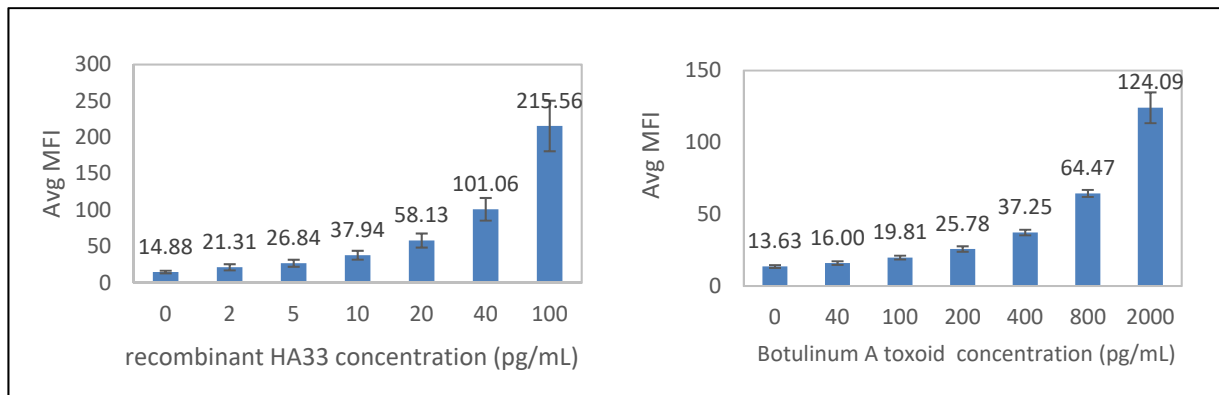


Figure 8. LOD refinement of HA33 and botulinum A toxoid.

LOD refinement of recombinant HA33 was performed between 2 and 100 pg/mL. Four separate runs of 24 replicates at each concentration of antigen were performed on two different MAGPIX instruments by two different operators on two days. The data presented in Table 2 are based upon 16 replicates generated at each test concentration.

Table 2. LOD Refinement of Recombinant HA33

Concentration (pg/mL)	Average MFI	%CV	SD	S/N
Background	14.88	12.09	1.80	1.00
2	21.31	19.89	4.24	1.43
5	26.84	18.06	4.85	1.80
10	37.94	16.18	6.14	2.55
20	58.13	16.67	9.69	3.91
40	101.06	15.42	15.59	6.79
100	215.56	16.10	34.72	14.49

CV, coefficient of variation.

SD, standard deviation.

LOD refinement of botulinum A toxoid was performed between 40 and 2000 pg/mL. Four separate runs of 24 replicates at each concentration of antigen were performed on two different MAGPIX instruments by two different operators on two days. The data presented in Table 3 are based upon 16 replicates generated at each test concentration.

Table 3. LOD Refinement of Botulinum A Toxoid

Concentration (pg/mL)	Average MFI	%CV	SD	S/N
Background	13.63	7.28	0.99	1.00
40	16.00	7.97	1.27	1.17
100	19.81	6.73	1.33	1.45
200	25.78	7.42	1.91	1.89
400	37.25	5.24	1.95	2.73
800	64.47	3.81	2.46	4.73
2000	124.09	8.62	10.70	9.11

4. CONCLUSIONS

In this study, large quantities of thermostable Rec α -MS2 and Rec α -HA33 were developed using transient transfections in mammalian cells. Antibody purifications were >95%, and post-production testing validated the purification activities and thermostability at 70 °C. In addition, sandwich-type immunoassays were developed using the MAGPIX platform. The recombinant and analogous polyclonal antibodies were provided by DBPAO. The results showed that Rec α -MS2 did not demonstrate superior activity than Rab α -MS2, which displayed poor binding affinity for intact MS2 bacteriophage. However, Rec α -MS2 did have confirmed affinity to the MS2 CP recombinant. The recombinant antibody was developed using recombinant MS2 CP as a target, so recombinant α -MS2 was less capable of recognizing CP as part of the intact bacteriophage. In contrast, Rec α -HA33 proved to be a strong capture and detector of antibodies in the MAGPIX sandwich immunoassay. Sensitivity for the recombinant HA33 assay, using

Rab α -BOT as the detector and Rec α -HA33 as the capture, achieved detection levels as low as picograms per milliliter. Until now, only recombinant protein and toxoid have been used in immunoassays on the MAGPIX platform, but more testing with active botulinum A toxin forms is required to complete assay development. Thermostable recombinant antibody products have attributes that will enable them to become part of the DBPAO repository at DEVCOM CBC.

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ACRONYMS AND ABBREVIATIONS

AP	A-protein
AR2G	amine reactive second-generation
ATP	Antibody Technology Program
BLI	biolayer interferometry
CP	coat protein
CV	coefficient of variation
DBPAO	Defense Biological Product Assurance Office
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
HA33	hemagglutinin 33
HC	heavy chain
IgG	immunoglobulin G
K _a	the binding constant
K _d	the dissociation constant
K _D	the equilibrium dissociation constant
LC	light chain
LOD	limit of detection
MFI	mean fluorescence intensity
NHS	<i>N</i> -hydroxysuccinimide
NHS-LC-LC-Biotin	succinimidyl-6-[biotinamido]-6-hexanamidohexanoate
PBS	phosphate-buffered saline
Rab α -BOT	rabbit anti-botulinum A
Rab α -MS2	Rabbit anti-MS2
Rec α -HA33	recombinant antibody to hemagglutinin 33
Rec α -MS2	recombinant antibody to MS2 bacteriophage
RNA	ribonucleic acid
SAPE	streptavidin-R-phycoerythrin
SD	standard deviation
S/N	signal to noise ratio

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