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ABERDEEN PROVING GROUND, MD 21010-5424

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Development of High-Protein-Producing Cell Lines from Hybridoma Clones Using Lentiviral Vectors

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14. ABSTRACT:

The objective of this report is to present data resulting from a successfully created mammalian cell line expressing *Bacillus anthracis* monoclonal antibodies (Mabs), which was obtained using the Lentigen system (Lentigen Corp.; Gaithersburg, MD). The Lentigen protein manufacturing platform uses a novel vector system to rapidly generate cell lines capable of producing proteins. The LentiMax lentiviral gene delivery technology can stably deliver one or more nucleic acid sequences (that encode proteins) to a mammalian cell with up to 100% efficiency. Multiple protein-producing mammalian cell lines can be created in as little as four weeks because the LentiMax system allows nucleic acid sequences under 6 kb in length to be rapidly cloned. Transduction of cells with lentiviral particles is simple and allows for high copy numbers from the gene of interest. Lentigen Corp. has demonstrated that its LentiMax technology can produce clonal cells that express secreted proteins of interest to an excess of 10 g/L. This would dramatically reduce the cost to produce valuable proteins such as Mabs. Lentigen Corp. has received a Small Business Technology Transfer (STTR) Program grant to continue this work with the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD).

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Lentivirus	*Bacillus anthracis* antibody
Lentigen Corporation	

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PREFACE

The work described in this report was authorized under project no. W911SR08-C-0080. This work was started in October 2007 and completed in September 2009. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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1. INTRODUCTION

One of our most pressing national defense concerns is the use of biological warfare for terrorism. A critical need in this area is the ability to quickly and accurately detect biological agents. The development of detection devices requires a means of identifying a particular agent by its distinguishing characteristics, which may include unique surface molecules, specific genetic sequences, or other markers. One of the biological agents of most concern is *Bacillus anthracis*, the causative agent of anthrax. Anthrax is primarily a disease of domesticated livestock, but it can also occur in humans and lead to rapid death. *B. anthracis* is a convenient and widely used bio-agent for warfare and terrorism because it can be grown easily and it forms spores. Spores represent a stable, metabolic form of the bacterium encased in a coat that protects it from extreme temperatures, chemicals, and physical damage. This makes the spore form of the organism suitable for incorporation into explosive weapons and concealment in terrorist devices.

To meet the detection capability requirement, hybridoma cell lines producing monoclonal antibodies (Mabs) that bind specifically to spores of *B. anthracis* were developed under a Technology Transition Program (*1*, *2*). The resulting hybridoma cell lines produced quantities of Mabs ranging from 29 to 600 mg per 3 L of supernatant. The Mabs were sent to outside laboratories for testing, and it was determined that two low-producing, related clones showed high specificity in diagnostic applications. Attempts to mass produce these antibodies by standard production methods (mouse ascites and cell culture) did not produce sufficient material; therefore, they were chosen as model hybridoma cell lines for Lentigen Corporation (Gaithersburg, MD) to develop into high-yielding clones. This included cloning out the sequence of the heavy and light chains, inserting these sequences into plasmids used to produce lentiviral particles, making lentiviral particles, transducing mammalian cells with the particles, and selecting stable mammalian cells to produce antibody at high yields and purity.

Lentigen Corp. has developed a protein manufacturing platform based on a novel vector system. The platform can efficiently generate cell lines capable of producing proteins at high yield and purity in a short time period. Lentigen Corp. uses its LentiMax lentiviral gene delivery technology to stably deliver one or more nucleic acid sequences (that encode proteins) to a mammalian cell with up to 100% efficiency. Multiple protein-producing mammalian cell lines may be created in as little as four weeks. The LentiMax system is inherently flexible and modular, which allows all nucleic acid sequences under 6 kilobases (kb) in length to be rapidly cloned. Transduction of cells with lentiviral particles is simple and allows for high copy numbers of the gene of interest. Theoretically, the transduced cell lines should be able to produce protein indefinitely. Lentigen Corp. received a Transfer Initiative Grant supported by the state of Maryland and entered a Cooperative Research and Development Agreement with the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) to develop high-producing cell lines from hybridomas. ECBC is now known as the U.S. Army Combat Capabilities Development Command Chemical Biological Center.

2. MATERIALS AND METHODS

2.1 Biological Materials

2.1.1 Hybridoma Cell Lines

Two anti-*B. anthracis* hybridoma cell lines, designated 5.1 and 5.2, were expanded from frozen stocks at ECBC. The cell lines were removed from the freezer and thawed in a 37 °C water bath. The recovered cells were resuspended in culture media (RPMI 1640 medium, 5% fetal bovine serum [FBS], 2 mM glutamine, 1 mM sodium pyruvate, 10 µM 2-mercaptoethanol, and 100 µg/mL each penicillin and streptomycin), counted, transferred to sterile culture flasks, and incubated at 37 °C with 5% CO_2. Fresh medium was added as required until flasks reached the log phase of growth. The cells were then counted and resuspended in freeze medium (90% FBS, 10% dimethyl sulfoxide) and transferred to a cryogenic freezer for freezing. Each of the clones was transferred to Lentigen Corp. for cloning.

2.1.2 Chinese Hamster Ovary (CHO) Cell Lines

The CHO K-1 cell line is a subclone of the original cell line derived from Chinese hamster ovaries. These mammalian cell lines are commonly used along with other methods for production of recombinant proteins.

2.1.3 293FT Cell Line

The 293FT cell line is derived from human embryonal kidney cells. They are fast growing and easily transducible, which makes them suitable for transduction by insertion of genetic material into the cell.

2.1.4 Antigens Used for Antibody Characterization

2.1.4.1 Whole Organism

B. anthracis delta Sterne (Ba) from existing ECBC stock was diluted for use in immunoassays.

2.1.4.2 Bacillus Exosporium Protein

Bacillus collagen-like protein of anthracis (BclA) is the antigen that was used for all testing. This protein is a major structural component of the spore-coat protein from *B. anthracis,* which has been shown to be highly immunogenic (*2*).

2.2 Cloning the Antibody Sequences at Lentigen Corp.

2.2.1 Heavy- and Light-Chain Cloning

Using standard polymerase chain reaction (PCR) technology, domain-specific primers were used to extract and amplify the heavy and light chains from the 5.1 and 5.2 clones. The resulting DNA was then separated by gel electrophoresis and sequenced to determine the specific nucleotide arrangement.

2.2.2 Plasmid Production Using Lentiviral Vectors

The two heavy-chain plasmids encoding the 5.1 and 5.2 clones and one κ-light-chain payload plasmid were used to build the three lentiviral vectors pLTG761, pLTG763, and pLTG735. Elongation factor (EF)-1α promoter vector (Clontech Laboratories; Mountain View, CA) was used in each vector at a titer of 10^8 multiplicity of infection per milliliter (MOI/mL). Vector particles were produced from each vector construct.

2.2.3 Cell Line Transductions

The vectors were initially used to transduce 293FT cells. Antibody from the clone was sent to ECBC for comparison with the native antibody from the original clones. Following verification of antibody binding, the industry standard CHO-K1 cell line was then used for transductions. Once successful immunoglobulin (Ig) production for the bulk transduced culture was verified, single cell cloning was performed. The isolated single cell clones were ranked according to their raw Ig production capability (5–40 mg/L), and the most promising ones (clones 9 and 28) were selected for further development.

2.3 Antibody Characterization at ECBC

2.3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were performed in triplicate using standard indirect assay techniques. Antigen was diluted (Ba at 5 µg/mL and BclA at 2 µg/mL) in phosphate-buffered saline (PBS) and used to coat the odd-numbered rows of three Nunc MaxiSorp 96-well plates (Thermo Scientific; Waltham, MA). Even-numbered rows were coated with an equivalent concentration of bovine serum albumin (Sigma-Aldrich; St. Louis, MO). The plates were 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. Antibody was diluted in PBS-T to 1 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 (1 h, 37 °C). After 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) one-component HRP substrate (KPL) was added to each well. After 20 min at 37 °C, the optical density (OD) at the 405 nm light wavelength was determined using a Synergy H4 hybrid multimode microplate reader (BioTek; Winooski, VT). Data analysis was performed using Prism software (GraphPad Software; La Jolla, CA).

2.3.2 Molecular Weight (MW) and Purity by Electrophoresis

MW and purity data were collected using an Experion automated electrophoresis station (Bio-Rad Laboratories; Hercules, CA). For Experion analysis, antibody was standardized to a final concentration of 1 mg/mL by diluting it in PBS and creating 20 mL aliquots for all

testing. The bovine γ-globulin (BGG) control and the samples were then processed. Briefly, a Pro260 microfluidic chip (Bio-Rad Laboratories) 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 dithiothreitol (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. As the lid was closed, the sample needles were lowered into the wells. The instrument was operated by the Experion software, and 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.

2.3.3 Kinetic Analysis by Surface Plasmon Resonance (SPR)

Using a ProteOn XPR36 system (Bio-Rad Laboratories) and standard amine coupling chemistry, 470 response units of BclA was immobilized, in pH 3.0 acetate buffer, to a ProteOn GLC sensor chip. The JC8 antibody (100 nM produced in hybridoma cells or 150 nM produced in CHO cells) was injected across the chip surface at a 100 µL/min flow rate in a threefold dilution series for 150 s. The reaction dissociated for 600 s, and the data were fit to a Langmuir 1:1 model using ProteOn Manager software.

2.3.4 Antibody Recognition by Western Blot Analysis

Samples were analyzed using a standard Western Blot protocol. Briefly, recombinant BclA protein was diluted in loading dye containing 2-mercaptoethanol (Sigma-Aldrich). These diluted samples were heated at 95 °C for 5 min, separated on a 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, and transferred to a nitrocellulose filter for detection. The membrane was blocked with 1× MDB and probed with either hybridoma-produced antibody or CHO cell-produced antibody for 1 h while it was rocked at room temperature. After it was washed with Tris-buffered saline with Tween (TBST; Sigma-Aldrich), the filter was again blocked with 1× MDB, and an HRP-labeled goat anti-mouse secondary antibody was added for 1 h while it was rocked at room temperature. The filter was then washed three times with TBST, developed using the ECL Prime western blotting detection reagent kit (GE Healthcare; Chicago, IL), and scanned on a Kodak imaging system (Eastman Kodak Co.; Rochester, NY).

3. RESULTS

3.1 Cell Line Production and Testing at Lentigen Corp.

3.1.1 Heavy- and Light-Chain Sequences from Cell Lines

With use of domain-specific primers, the heavy and light chains were extracted from the 5.1 and 5.2 clones. As expected, there was only one band representing the κ-light chain of the hybridoma, but two heavy-chain bands showed up in gel separating the PCR product (Figures 1–6). This indicated that the hybridoma contained at least one more version of Ig.

Sequencing data on the monoclonal isolates of the heavy-chain alleles showed that there were only two different clones. The homology between the two protein sequences derived from the consensus sequences was only 86.2%. The difference between the two chains was restricted to the variable region (Figures 4–6). Compared to the 5.1 clone, the 5.2 clone contained 12 base-pair insertions and over 30 base changes that resulted in differences in amino acid sequences.

caaataatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaaaaaagcaggctccgaattcgcccttcac
cATGGGATGGAGCTGGATCTTTCTCTTTCTCCTGTCAGGAACTGCAGGTGTCCTCTCTGAGGTCCAGCT
GCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGACTTCTGGAT
ACACATTCACTGAATACACCATGCACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGG
AGGTATTAATCCTAACAATGGTGGTACTAGCTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTG
TAGACAAGTCCTCCAGCACAGCCTACCTGGAGCTCCGCAGCCTGACATCTGAGGATTCTGCAGTCTAT
TACTGTGCAAGAGATGATAGGTACCCCGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGT
CTCTGCAGCCAAAACAACACCCCCATCAGTCTATCCACTGGCCCCTGGGTGTGGAGATACAACTGGTT
CCTCCGTGACTCTGGGATGCCTGGTCAAGGGCTACTTCCCTGAGTCAGTGACTGTGACTTGGAACTCTG
GATCCCTGTCCAGCAGTGTGCACACCTTCCCAGCTCTCCTGCAGTCTGGACTCTACACTATGAGCAGCT
CAGTGACTGTCCCCTCCAGCACCTGGCCAAGTCAGACCGTCACCTGCAGCGTTGCTCACCCAGCCAGC
AGCACCACGGTGGACAAAAAACTTGAGCCCAGCGGGCCCATTTCAACAATCAACCCCTGTCCTCCATG
CAAGGAGTGTCACAAATGCCCAGCTCCTAACCTCGAGGGTGGACCATCCGTCTTCATCTTCCCTCCAA
ATATCAAGGGTGTACTCATGATCTCCCTGACACCCAAGGTCACGTGTGTGGTGGTGGATGTGAGCGAG
GATGACCCAGACGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAA
CCCATAGAGAGGATTACAACAGTACTATCCGGGTGGTCAGCACCCTCCCCATCCAGCACCAGGACTGG
ATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCATCACCCATCGAGAGAACCA
TCTCAAAAATTAAAGGGCTAGTCAGGGCTCCACAAGTATACATCTTGCCGCCACCAGCAGAGCAGTTG
TCCAGGAAAGATGTCAGTCTCACTTGCCTGGTCGTGGGCTTCAACCCTGGAGACATCAGTGTGGAGTG
GACCAGCAATGGGCATACAGAGGAGAACTACAAGGACACCGCACCAGTCCTGGACTCTGACGGTTCT
TACTTCATATATAGCAAGCTCAATATGAAAACAAGCAAGTGGGAGAAAACAGATTCCTTCTCATGCAA
CGTGAGACACGAGGGTCTGAAAAATTACTACCTGAAGAAGACCATCTCCCGGTCTCCGGGTAAATGAta
aaagggcgaattcgacccagctttcttgtacaaagttggcattataaaaaataattgctcatcaatttgttgcaacgaacaggtcactatcagtcaaaataaaatcattatttg

Domain specific primers (attL1 and attL2) are in green
Signal peptide is in blue
Variable domain is in dark red
Constant domain is in light red

Figure 1. Heavy-chain 1 (5.1 clone). Full IgG heavy-chain sequence is in uppercase.

caaataatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaaaaaagcaggctccgaattcgcccttcac
cATGAAATGCAGCTGGATTATGTTCTTCCTGATGGCAGTGGTTATAGGAATCAATTCAGAGGTTCAGCT
GCAGCAGTCTGGGGCAGAGCTTGTGAGGTCAGGGGCCCCAGTCAAGTTGTCCTGCACAGCTTCTGGCT
TCAACATTAAAGACTACTATATGCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGG
ATGGATTGATCCTGAGAATGGTGATACTGAATATGCCCCGAATTTCCAGGGCAAGGCCACTATGACTG
CAGACACATCCTCCAACACAGCCTACCTGCAGTTCAGCAGCCTGACATCTGAGGACACTGCCGTCTAT
TACTGTAATGCCCATGTTTTATTACTACGGGGAGTAGAGGATGCTATGGACTACTGGGGTCAAGGAAC
CTCAGTCACCGTCTCCTCAGCCAAAACAACAGCCCCACCCGTCTATCCACTGGCCCCTGGGTGTGGAG
ATACAACTGGTTCCTCCGTGACTCTGGGATGCCTGGTCAAGGGCTACTTCCCTGAGTCAGTGACTGTGA
CTTGGAACTCTGGATCCCTGTCCAGCAGTGTGCACACCTTCCCAGCTCTCCTGCAGTCTGGACTCTACA
CTATGAGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGCCAAGTCAGACCGTCACCTGCAGCGTTGCT
CACCCAGCCAGCAGCACCACGGTGGACAAAAAACTTGAGCCCAGCGGGCCCATTTCAACAATCAACC
CCTGTCCTCCATGCAAGGAGTGTCACAAATGCCCAGCTCCTAACCTCGAGGGTGGACCATCCGTCTTC
ATCTTCCCTCCAAATATCAAGGATGTACTCATGATCTCCCTGACACCCAAGGTCACGTGTGTGGTGGTG
GATGTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAG
CTCAGACACAAACCCATAGAGAGGATTACAACAGTACTATCCGGGTGGTCAGCACCCTCCCCATCCAG
CACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCATCACCCA
TCGAGAGAACCATCTCAAAAATTAAAGGGCTAGTCAGAGCTCCACAAGTATACATCTTGCCGCCACCA
GCAGAGCAGTTGTCCAGGAAAGATGTCAGTCTCACTTGCCTGGTCGTGGGCTTCAACCCTGGAGACAT
CAGTGTGGAGTGGACCAGCAATGGGCATACAGAGGAGAACTACAAGGACACCGCACCAGTCCTGGAC
TCTGACGGTTCTTACTTCATATATAGCAAGCTCAATATGAAAACAAGCAAGTGGGAGAAAACAGATTC
CTTCTCATGCAACGTGAGACACGAGGGTCTGAAAAATTACTACCTGAAGAAGACCATCTCCCGGTCTC
CGGGTAAATGAtaaaagggcgaattcgacccagctttcttgtacaaagttggcattataaaaaataattgctcatcaatttgttgcaacgaacaggtcactatcagt
caaaataaaatcattatttg

Domain specific primers (attL1 and attL2) are in green
Signal peptide is in blue
Variable domain is in dark red
Constant domain is in light red

Figure 2. Lentigen 5.2 heavy-chain plasmid. Full IgG heavy-chain sequence is in uppercase.

caaataatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaaaaaagcaggctccgaattcgcccttcac
cATGAGGGCCCCTGCTCAGTTTCTTGGGTTCTTGTTGCTCTTGTTTCCAGGTACCAGATGTGACATCCAG
ATGACCCAGTCTCCATCCTCCTTATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGT
CAGGACATTGGTAGTAACTTAAACTGGCTTCAGCAGGAACCAGATGGAACTATTAAACGCCTGATCTA
CGCCACATCCAGTTTAGATTCTGGTGTCCCCAAAAGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATTC
TCTCACCATCAGCAGCCTTGAGTCTGAAGATTTTGTAGACTATTACTGTCTACAATATGCTAGTTCTCC
TCCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAACTGTATCCATCT
TCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACC
CCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTG
GACTGATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAG
TATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAGAG
CTTCAACAGGAATGAGTGTTGAtaaaagggcgaattcgacccagctttcttgtacaaagttggcattataaaaaataattgctcatcaatttgttgcaac
gaacaggtcactatcagtcaaaataaaatcattatttg

Domain specific primers (attL1 and attL2) are in green
Signal peptide is in blue
Variable domain is in dark red
Constant domain is in light red

Figure 3. Lentigen 5.1 light-chain plasmid. Full Ig κ-light-chain sequence is in uppercase.

ATGAGGGCCCCTGCTCAGATTTTTGGGTTCTTGTTGCTCTTGTTTCCAGGTACCAGATGTGACATCCAG
ATGACCCAGTCTCCATCCTCCTTATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAAGT
CAGGACATTGGTAGTAACTTAAACTGGCTTCAGCAGGAACCAGATGGAACTATTAAACGCCTGATCTA
CGCCACATCCAGTTTAGATTCTGGTGTCCCCAAAAGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATTC
TCTCACCATCAGCAGCCTTGAGTCTGAAGATTTTGTAGACTATTACTGTCTACAATATGCTAGTTCTCC
TCCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAAATGTATCCATCT
TCCCACCATCCAGTAAGCTTGGG

Figure 4. Light chain (variable).

TTGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAGTAAGCAAACCAGGCGGGGTACCTATCATCTCTT
GCACAGTAATAGACTGCAGAATCCTCAGATGTCAGGCTGCGGAGCTCCAGGTAGGCTGTGCTGGAGG
ACTTGTCTACAGTCAATGTGGCCTTGCCCTTGAACTTCTGGTTGTAGCTAGTACCACCATTGTTAGGAT
TAATACCTCCAATCCACTCAAGGCTCTTTCCATGGCTCTGCTTCACCCAGTGCATGGTGTATTCAGTGA
ATGTGTATCCAGAAGTCTTGCAGGATATCTTCACTGAAGCCCCAGGCTTCACCAGCTCAAGTCCAGAC
TGCTGCAGCTGGACCT

Figure 5. Heavy chain (variable) 5.1.

ATGGGATGGAGCTGGATCTTTCTCTTTCTCCTGTCAGGAACTGCAGGTGTCCTCTCTGAGGTCCAGCTG
CAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGACTTCTGGATA
CACATTCACTGAATACACCATGCACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGA
GGTATTAATCCTAACAATGGTGGTACTAGCTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTGT
AGACAAGTCCTCCAGCACAGCCTACCTGGAGCTCCGCAGCCTGACATCTGAGGATTCTGCAGTCTATT
ACTGTGCAAGAGATGATAGGTACCCCGCCTGGTTTGCTTACTGGGGCCAAGGGACCACGGTCACCGTC
TCCTCA

Figure 6. Heavy chain (variable) 5.2.

3.1.2 Plasmid Production

The two heavy-chain plasmids encoding the 5.1 and 5.2 clones and the one κ-light-chain payload plasmid were used to build the three lentiviral vectors pLTG761, pLTG763, and pLTG735, as shown in Figures 7–9.

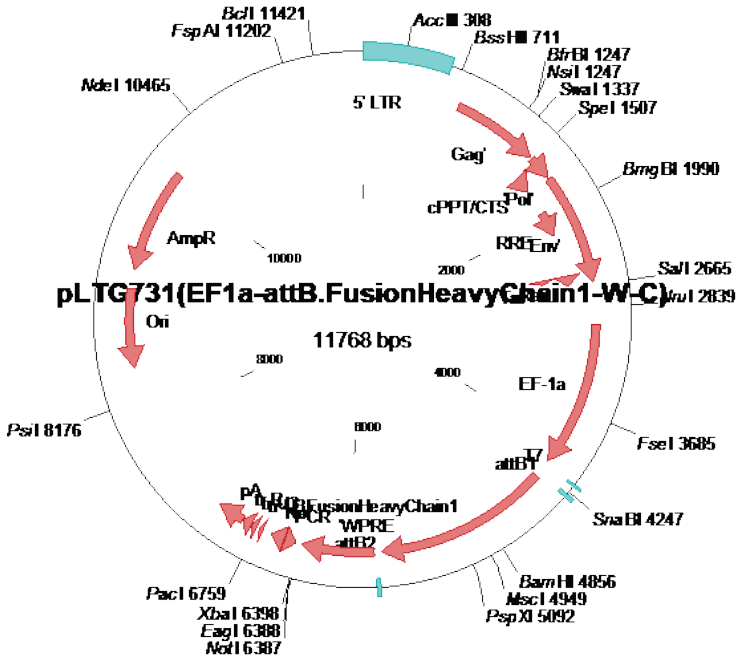


Figure 7. Heavy-chain plasmid pLTG731 for 5.1 clones.

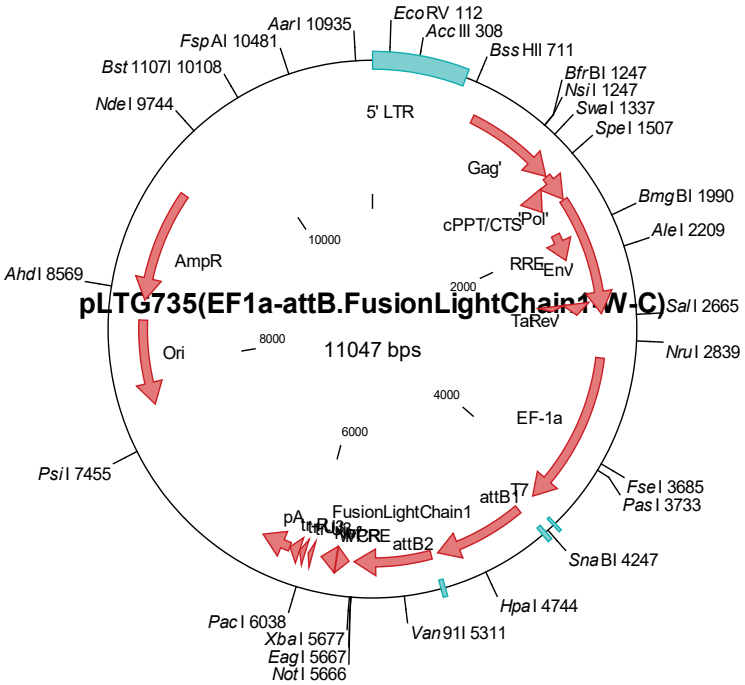


Figure 8. Heavy-chain plasmid pLTG733 for 5.2 clones.

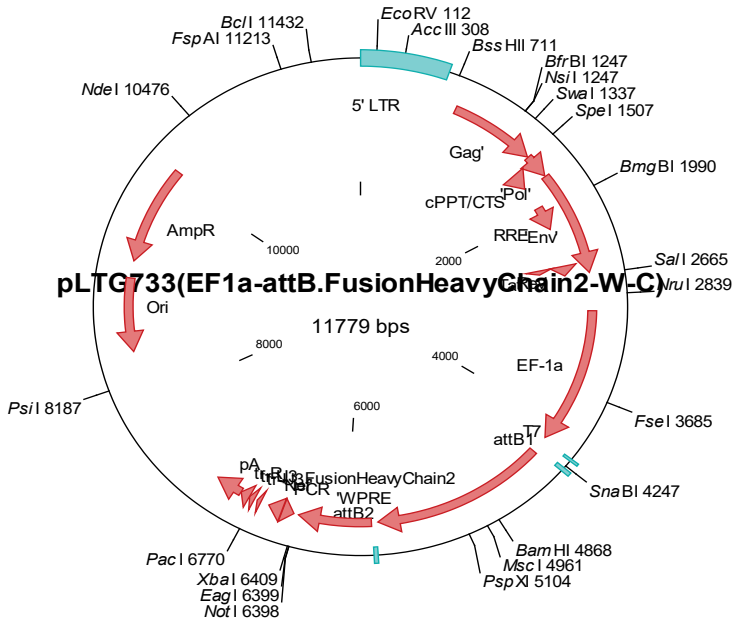


Figure 9. Common light-chain vector pLTG7351 for 5.1 and 5.2 clones.

3.1.3 Transduction of Cell Lines

Initial IgG levels produced in the supernatant of 293FT-transduced cells were obtained using IgG quantization, which shows the production of IgG. Once it was established that IgG was produced and that it bound the antigen, the project was reinitialized in CHO-K1 cells using the modified lentiviral construct (Figure 10).

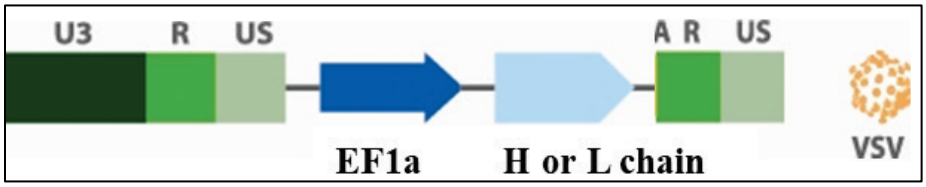


Figure 10. Lentiviral construct. Modified lentiviral construct containing the EF1-α promoter and either the heavy (H) or light (L) chain.

3.1.4 Supernatant Testing

The initial supernatant from transduction 2 was sent to ECBC for further analysis. A summary of the Army results is shown in Figure 11. The antibody was found to be specific for *B. anthracis,* and the antibody concentration was about 5 µg/mL.

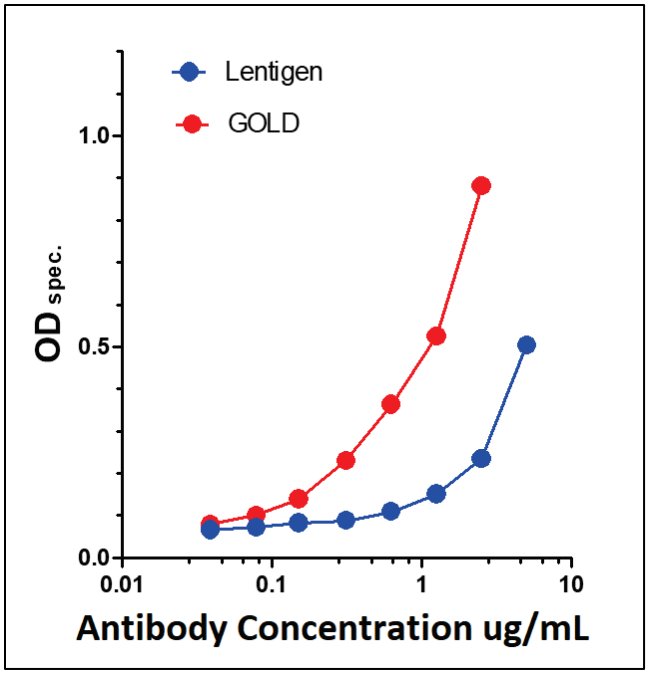


Figure 11. Functional comparison of supernatant for transduction 2 to "gold standard" Mab. The bulk cell culture supernatant was collected after two transductions. The isotype of the antibodies was IgG2b with κ-light chain.

3.1.5 Clone Selection and Testing

The bulk culture of 5.1-transduced cells was single-cell cloned, and the clones were tested for Ig production. The scale of raw Ig production allowed us to rank the clones initially and select the best producing clones for further analysis and optimization (Figure 12). Production levels up to 40 mg/L were achieved in the clones, and the growth characteristics of the most promising cell lines were characterized.

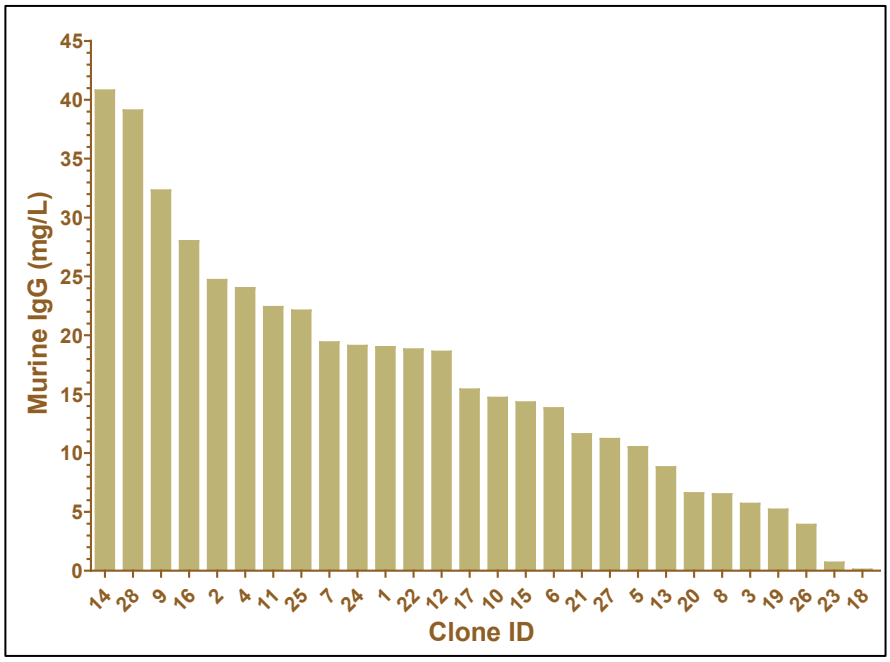


Figure 12. Distribution of Ig production in representative clones of 5.1 IgG.

Bulk culture 5.2 produced 1–5 mg/L of Ig under non-optimized conditions. The cells were single-cell cloned. After 562 clones were tested, 28 were selected for further testing. After retesting, 6 clones were chosen for further assays, as shown in Figure 13.

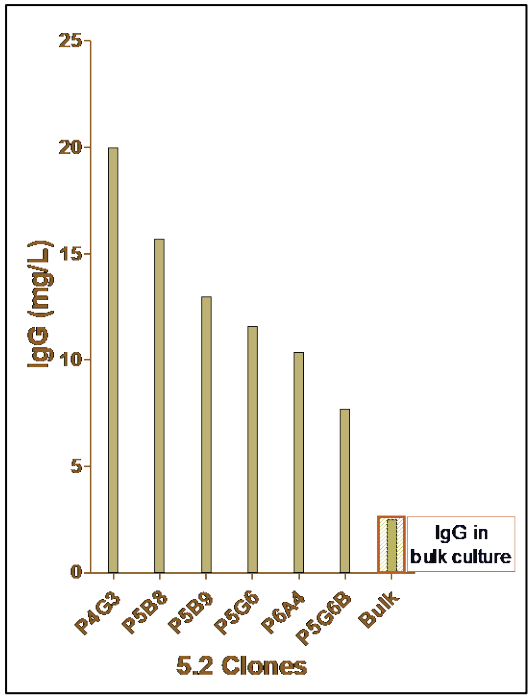


Figure 13. Selected clones from the bulk 5.2 culture.

All selected clones were cryo-preserved and stored in liquid nitrogen. Supernatants were collected and are ready for functional analysis.

3.1.6 Clonal Analysis of 5.1 and 5.2 Igs

Two clones for each group were analyzed in Western blots to determine the expression and secretion of intact Igs into supernatant (2H + 2L). It was very encouraging that we did not see substantial accumulation of Ig degradation products and the secreted Ig (Figure 14).

However, the IgG heavy chain from the cell lysate had MWs of 54–58 kDa, as compared to the heavy chain from the secreted Ig, which had the nominal MW of 50 kDa (1H). The light chain had the standard MW of 25 kDa in all cases, and it looked normal.

In both of the 5.2 clones, the heavy chains (1H) with increased MW were accumulated in the cytoplasm. This form, which assembled into 1H+1L complexes, was apparently less capable of assembling into full IgGs consisting of 2H+2L chains. Furthermore, the secretion from 5.1-P4A8 cells was very efficient, whereas the 5.2 clones had a far lower apparent ratio of secreted or intracellular IgG (Figure 15).

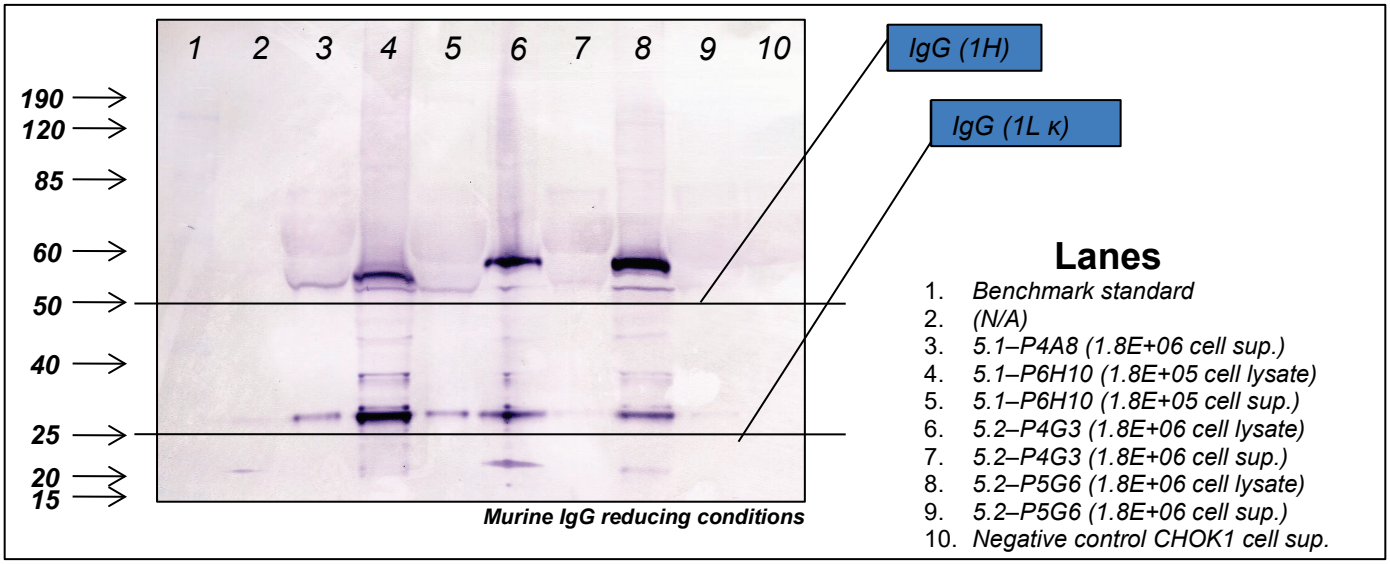


Figure 14. Western blot from denaturing reducing gel.

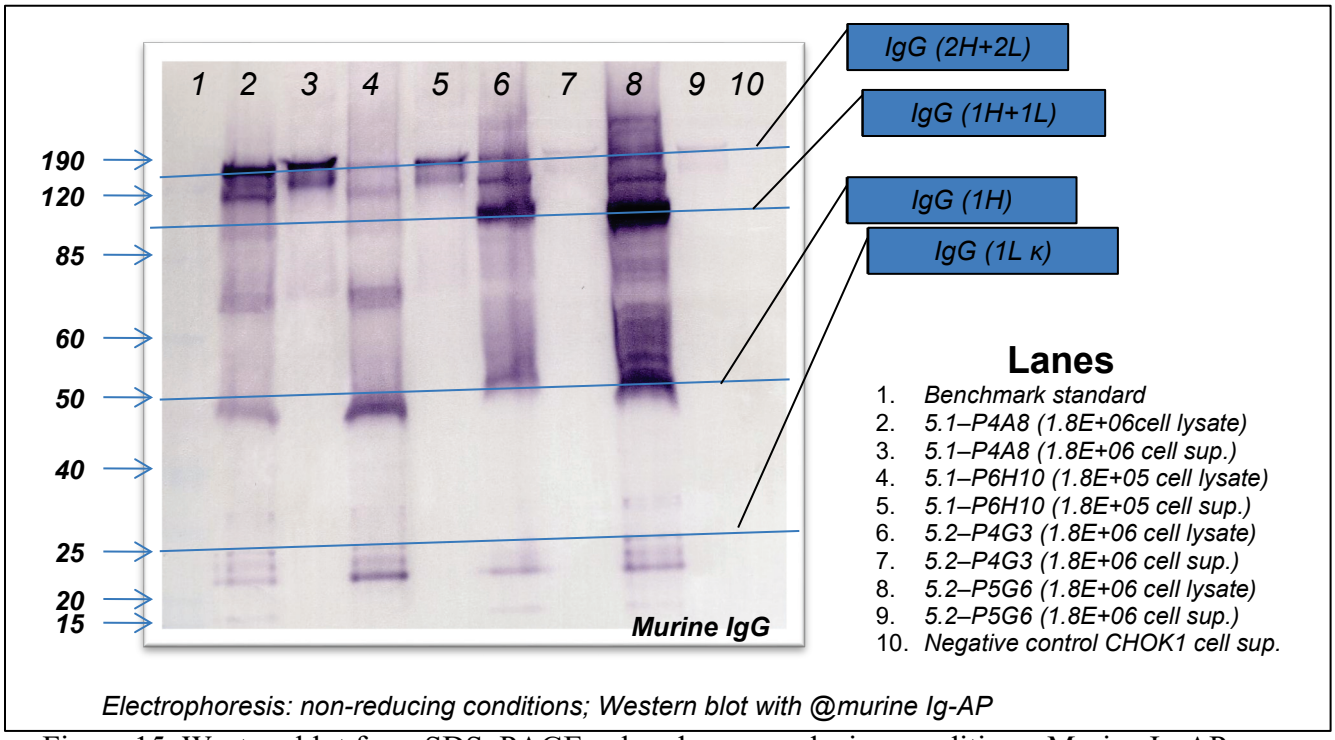


Figure 15. Western blot from SDS–PAGE gel under non-reducing conditions. Murine Ig-AP, alkaline phosphatase-linked mouse IgG.

3.2 Characterization of Purified Antibody at ECBC

3.2.1 ELISA

Antibodies from the clones were tested against the native antibody using a whole organism and the BclA surface protein as described in Section 2.3.1. Figure 16 shows that both antibodies reacted similarly to the different antigens.

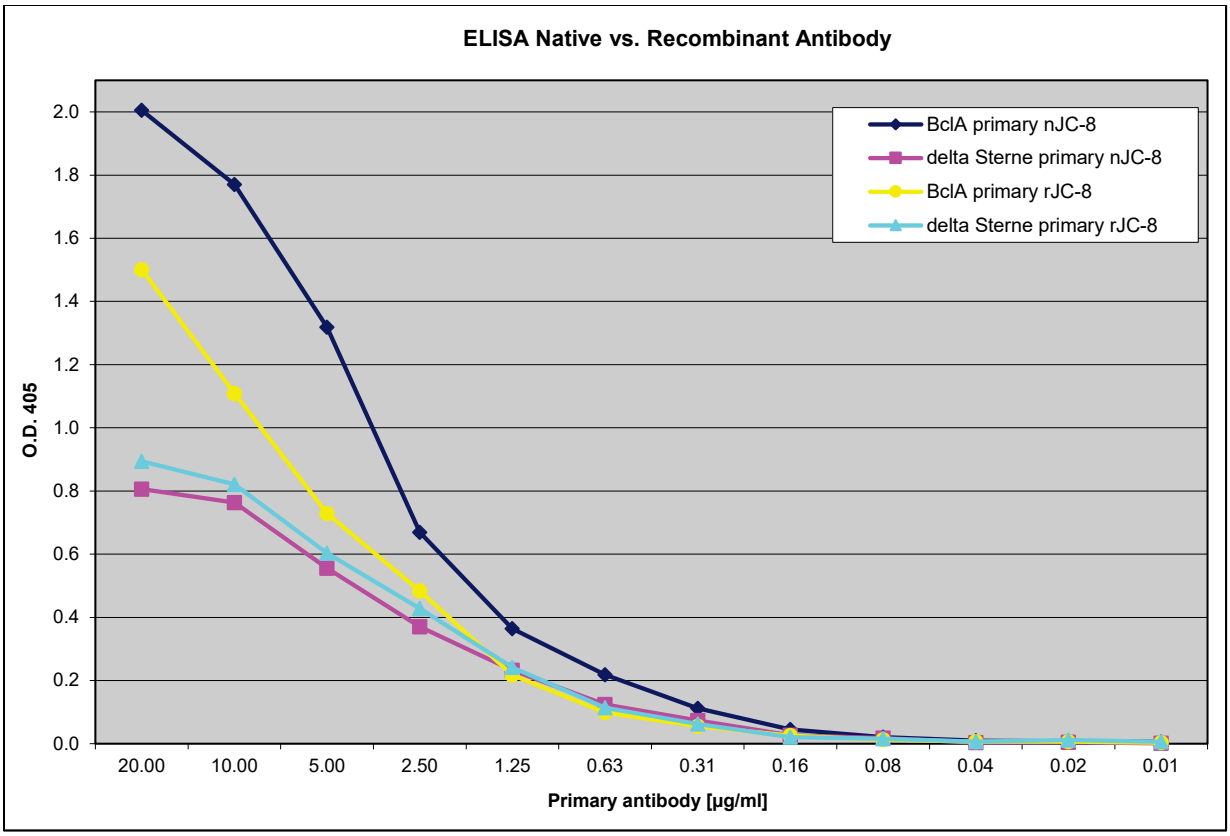


Figure 16. Standard ELISAs were performed to compare the reactivity of the native antibody (nJC-8) with the recombinant antibody (rJC-8) produced in the CHO cells. The antibodies reacted similarly to one another.

3.2.2 MW and Purity

The MW of the antibody from clones 9 and 28 was determined using the Experion Pro260 analysis kit, as shown in Figure 17. The thick bands at the top of the second and third lanes correspond to the antibody heavy chain. The lower band is the light chain. According to the software, the antibodies were determined to have MWs of 32.8 and 65.5 kDa, and the purity was 97% for each clone.

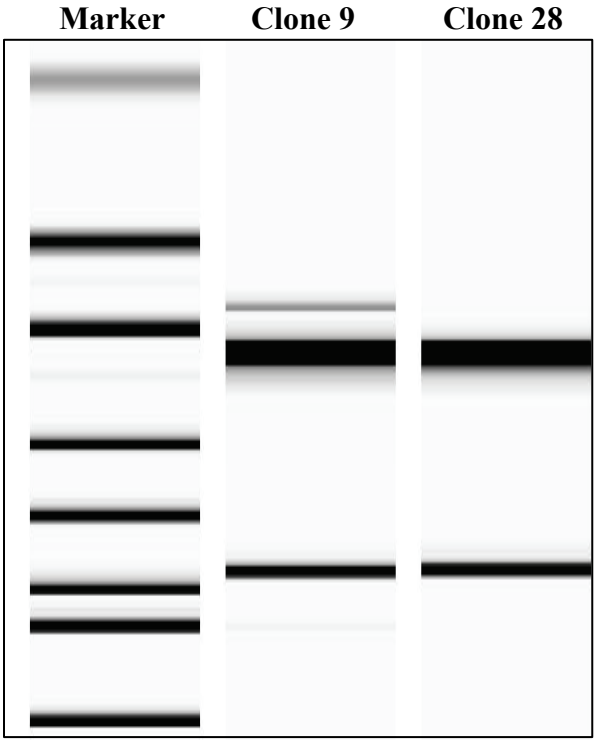


Figure 17. Clones from Lentigen Corp. were run on the Experion system, using the Pro260 analysis kit. Antibodies were determined to have MWs of 32.8 and 65.5 kDa, and the purity was 97% for each clone.

3.2.3 Western Blot Analysis

Standard Western blots were performed to compare the reactivity of the native antibody (hybridoma) with the recombinant antibody produced in CHO cells. Figure 18 shows that the antibodies reacted remarkably similar to one another.

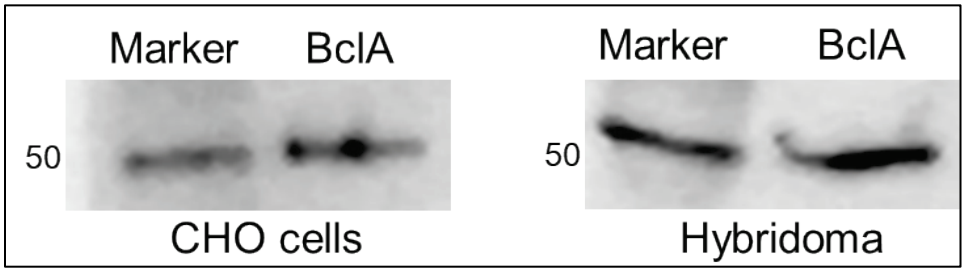
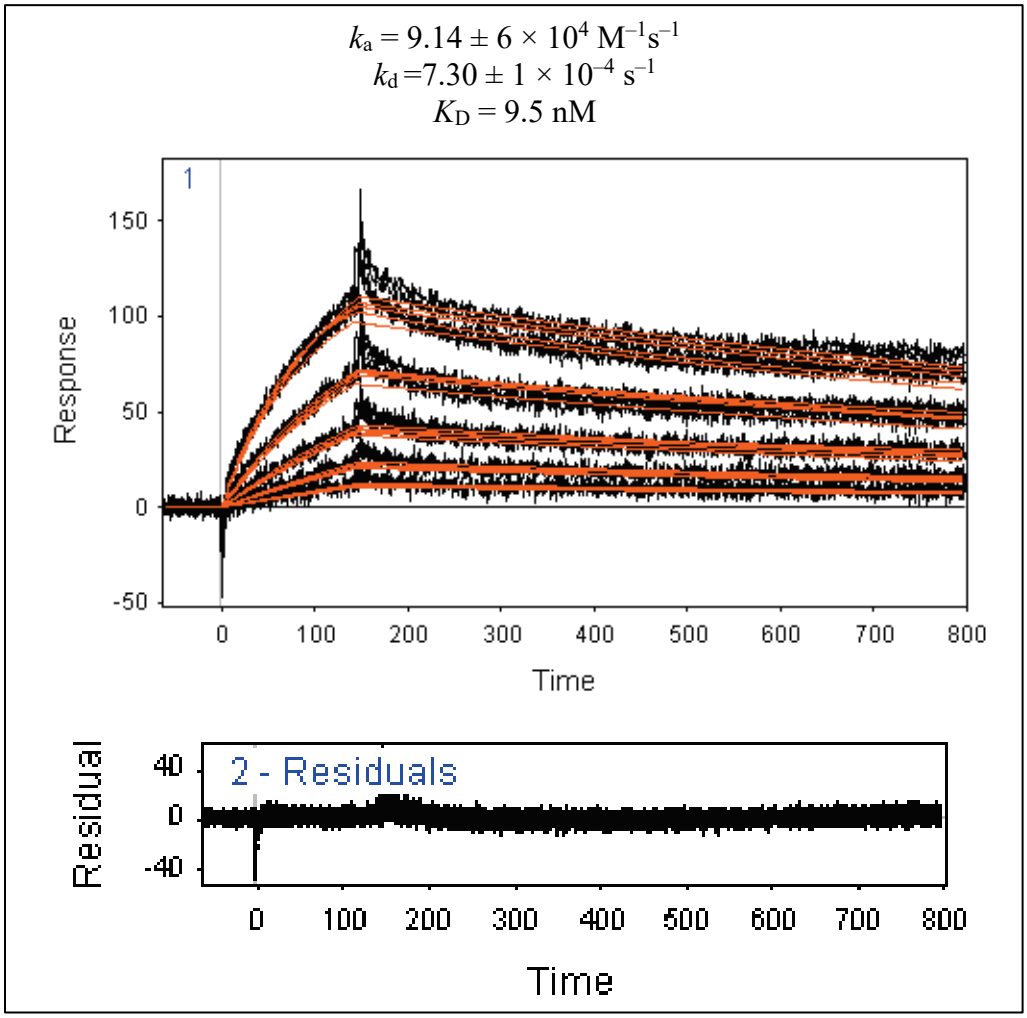


Figure 18. Western blots of CHO cell antibody compared with native antibody.

3.2.4 Kinetic Analysis

Kinetic analysis of the binding of antibodies to the BclA antigen was performed as a direct binding SPR experiment on the ProteOn XPR36 system. Results are presented in Figure 19. Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using the ProteOn XPR36 software (Bio-Rad). The equilibrium constant (K_D) of the native antibody was determined to be 9.5 nM. Results from similar experiments conducted using the recombinant antibody are also presented in Figure 19. The K_D of the recombinant antibody was determined to be 9.3 nM, thereby showing the similarity of the two binding characteristics.



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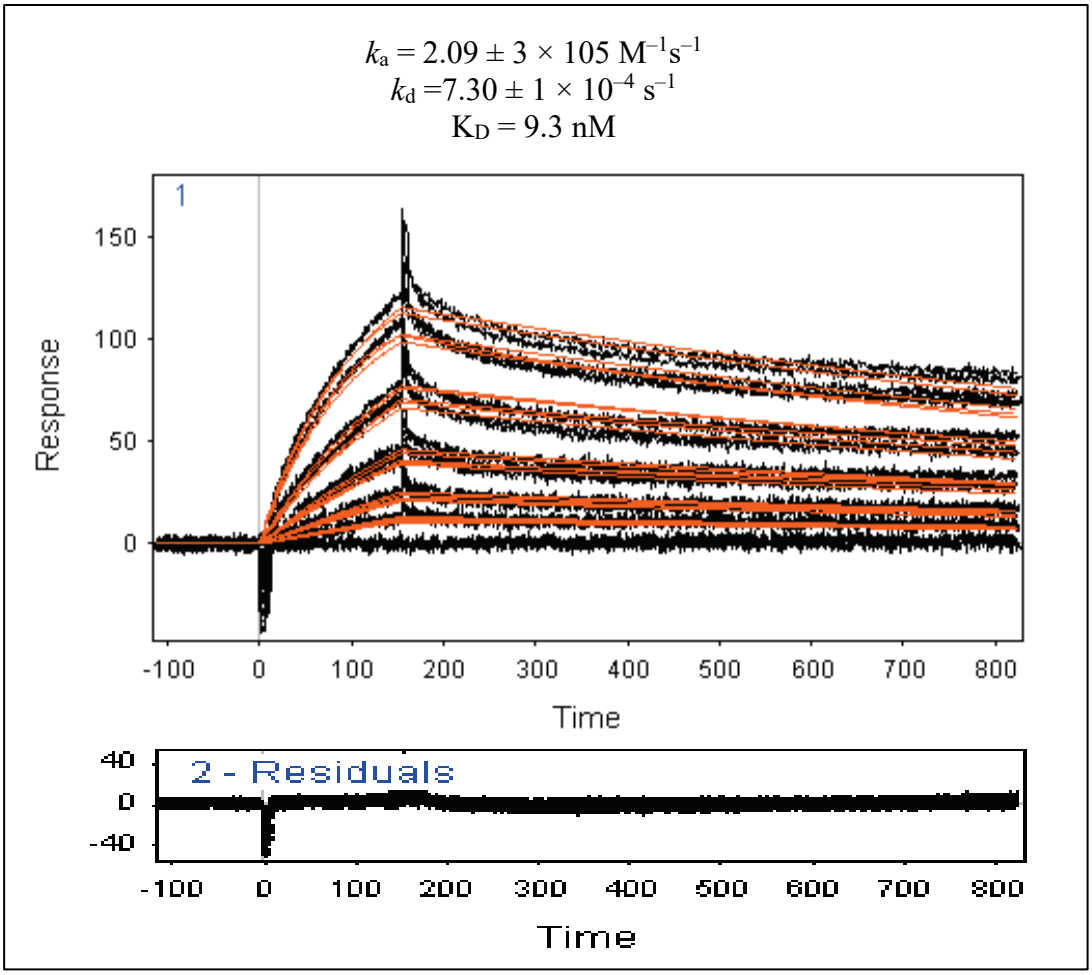

Figure 19. Kinetic analysis of the native antibody from hybridoma (top) and the recombinant antibody from CHO cells (bottom). Standard SPR was performed to compare the reactivity of the native antibody (hybridoma) with that of the recombinant antibody produced in CHO cells. Both antibodies reacted similarly to one another, and the K_D values were 9.5 and 9.3 nM, respectively. k_a and k_d, association and dissociation constants, respectively.

4. CONCLUSIONS

Production methods for antibodies used in detection devices have drastically changed over time. Animal models provided the most common method; however, they produced polyclonal antibodies that lacked antigen binding specificity, and the antibody affinity depended on individual animals. Development of the Mab gave the assays more specificity, but the use of a large number of animals was still required. 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 have greatly increased the ability of researchers to produce more specific antibodies with antigen binding fragments (Fab), single-chain variable fragments (scFv), and single-domain antibodies (sdAb). Many varying

recombinant production systems are being used, from basic bacteria, yeasts, and filamentous fungi to insect cell lines and mammalian cells, including transgenic plants and animals (*1*). This report shows the benefits of using alternative production methods to increase yield without altering the affinity of the antibodies.

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ACRONYMS AND ABBREVIATIONS

Ba	*Bacillus anthracis* delta Sterne
BclA	bacillus collagen-like protein of anthracis
BGG	bovine γ-globulin
CHO	Chinese hamster ovary
ECBC	U.S. Army Edgewood Chemical Biological Center
EF	elongation factor
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
HRP	horseradish peroxidase
Ig	immunoglobulin
K_D	equilibrium constant
Mabs	monoclonal antibodies
MDB	milk diluent block
MOI	multiplicity of infection
MW	molecular weight
OD	optical density
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20
PCR	polymerase chain reaction
scFv	single chain variable fragment
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TBST	Tris-buffered saline with Tween

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