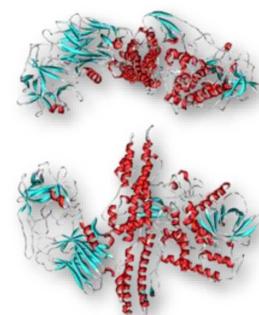


## Abstract

Recombinant  $\alpha$ -HA33 IgG antibody was developed using transient transfections in HEK-293F suspension cells. Transfections were performed using different conditions to determine optimal yields of light and heavy chain, as well as an optimal time course for antibody production. The project will further the development of recombinant antibodies as improved reagents, establishing optimal antibody production protocols and validating ISO Guide 34 quality standards.

## Background



Botulinum neurotoxin (BoNT) poisoning primarily occurs from the accidental ingestion of *Clostridium botulinum* contaminated food. BoNTs inhibit the release of neurotransmitters, which leads to muscle paralysis. Hemagglutinin 33 (HA33), a surface protein believed to bind to host proteins and cell surface molecules, is thought to be critical in toxin internalization, protection, and most likely, activation of the toxin.

This study represents a transition from Defense Advance Research Projects Agency (DARPA) Antibody Technology Program (ATP), with the objective of developing an  $\alpha$ -HA33 antibody in a mammalian cell line. Now development has turned its focus on the optimization of antibody production geared toward large scale manufacturing. If successful, the product will become part of the Critical Reagents Program (CRP) repository at the Army's Edgewood Chemical and Biological Center (ECBC). The antibody can be used in a variety of assay formats with broad applications in point detection of toxin in environmental samples, medical diagnostics and food safety – all of which can benefit the Soldier and provide capabilities that shape and sense field operations.

## Materials and Methods

### HEK-293F Cell Culture

Human Embryonic Kidney (HEK) cells designated Expi293F (Life Technologies) were thawed and grown in 30 mL Erlenmeyer Flasks in Expi293 Expression Medium (Life Technologies) at 37°C, 8% CO<sub>2</sub>, and 195 rpm. Cells were passaged once they reached 5x10<sup>6</sup>/ml, and new flasks were seeded at 4x10<sup>5</sup>/ml. Cells were discarded once they reached passage 20.

### Transient Transfection

Cells were transfected once they reached a cell density of 2.5x10<sup>6</sup>/ml and were more than 95% viable. All reagents and protocols were taken from Life Technologies ExpiFectamine 293 Transfection Kit. Briefly, DNA and media were mixed with ExpiFectamine 293 reagent and incubated for 30 minutes to form DNA-lipid complexes. Complexes were then added to cell suspensions in 24-well plates under variable transfection conditions. At 18 hours post-transfection, Enhancer 1 and 2 mixture was added to each well.

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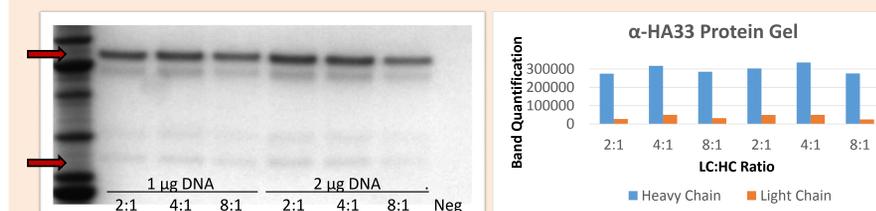
### Affinity Purification

All sample supernatants were collected and subjected to protein A affinity purification. Protein A HP SpinTrap columns (GE Healthcare Life Sciences) were equilibrated in neutral pH phosphate buffer. Supernatants were applied, and IgG was eluted with pH 2.7 (glycine buffer), and neutralized with 1M Tris-HCl buffer with pH 9.0.

### Optimization of DNA Concentration and Expression Yield

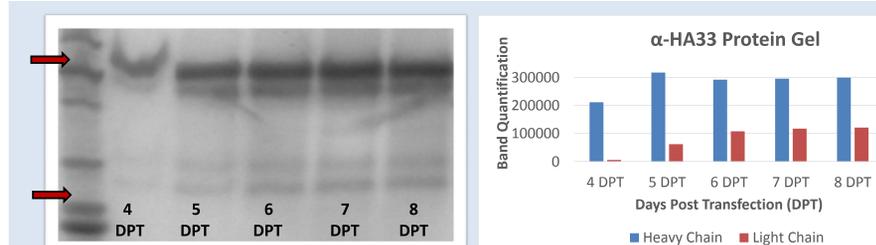
Cells were co-transfected with two plasmids, one coding for the light chain (LC) and the other coding for the heavy chain (HC) of the  $\alpha$ -HA33 IgG. Variable amounts of DNA (1-2  $\mu$ g/1 mL transfection) and variable ratios of LC chain to HC, ranging from 2:1 to 40:1, were tested to determine the requirements for optimal expression. Once the optimal DNA ratio was determined, transfected cells were sampled from 30 hours to 8 days post-transfection to identify the incubation time yielding the maximum quantity of the  $\alpha$ -HA33 IgG protein.

### Transfection Optimization



**Figure 1. Left:** SDS PAGE image from 1 mL transfection optimization. Top arrow marks the molecular weight of the HC. Bottom arrow marks the molecular weight of the LC. **Right:** Bands were analyzed using LI-COR Image Studio software. All shown samples were collected 4 days post-transfection. The sample using 1  $\mu$ g total DNA and 4:1 LC to HC ratio was selected for the time course study due to two factors: the highest total amount of LC produced and the highest proportion of LC to HC produced (16% versus 15% for the 2  $\mu$ g total DNA and 4:1 LC to HC ratio.) It should be noted that transfections with higher ratios of LC DNA showed little to no production of light chain (data not shown).

### Time Course Optimization

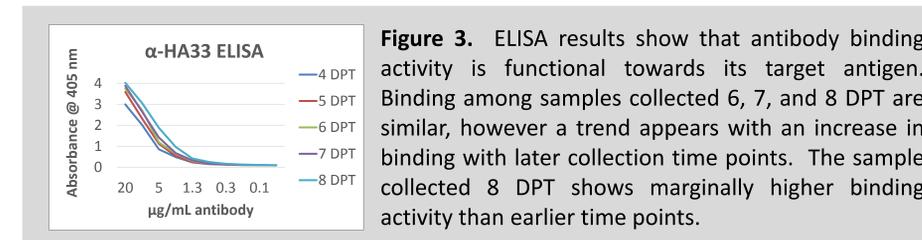


**Figure 2. Left:** SDS PAGE image from the time course optimization. From left to right; BenchMark Protein Ladder (Life Technologies),  $\alpha$ -HA33 4, 5, 6, 7, and 8 days post transfection (DPT). Top arrow marks the molecular weight of the HC. Bottom arrow marks the molecular weight of the LC. **Right:** Bands were analyzed using LI-COR Image Studio software. Although samples collected 6, 7, and 8 DPT were very similar, the sample collected at 8 DPT exhibited the highest amount of LC produced, and the highest proportion of LC to HC produced (40% versus 37% and 39% in the 6 and 7 DPT samples respectively).

## Results

### Validation of IgG Activity

An ELISA was performed using purified  $\alpha$ -HA33 antibody from the time course study. A 96-well-plate was coated with 2  $\mu$ g/ml  $\alpha$ -HA33 protein antigen (ECBC) in PBS. The plate was stored at 4°C overnight. The following morning the plate was washed 3 times using KPL wash buffer using an AquaMax2000 plate washer. The plate was then blocked for 30 minutes at room temperature with KPL milk-based block. The plate was washed and  $\alpha$ -HA33 antibodies (purified from the time course experiment samples, see Fig. 2) were added at 20  $\mu$ g/ml in blocking solution with 1:2 serial dilutions. The primary antibody was incubated for 60 minutes at room temperature before the plate was washed again. KPL goat anti-human secondary antibody was then added at 1:2500 in blocking solution and incubated at room temperature for 60 minutes. The plate was then washed and developed using ABTS (KPL) substrate. The plate was read at 405 nm on a Biotek plate reader and data was analyzed using Excel.



**Figure 3.** ELISA results show that antibody binding activity is functional towards its target antigen. Binding among samples collected 6, 7, and 8 DPT are similar, however a trend appears with an increase in binding with later collection time points. The sample collected 8 DPT shows marginally higher binding activity than earlier time points.

## Conclusion

In this study, conditions to generate optimal production and activity of recombinant  $\alpha$ -HA33 IgG were investigated using transient transfection of HEK-293F cells. While most experimental conditions investigated favored the production of IgG's HC, production of the IgG's LC proved to be significantly lower. One  $\mu$ g of DNA with a ratio of 4:1 LC to HC produced the highest quantity of LC and the highest proportion of LC to HC. Allowing the cells to incubate for up to 8 DPT produced the most amount of LC and the highest proportion of LC to HC, though the differences between 6, 7 and 8 day incubations were nominal. The ELISA assay also showed that the recombinant expression of  $\alpha$ -HA33 is functionally reactive towards its target antigen. More research is required to understand the variables involved in controlling LC versus HC production and validating the incubation conditions for optimizing production and assemblage of the recombinant  $\alpha$ -HA33 IgG in larger scale productions.



## Acknowledgments

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