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Matt Abbott

Jacob Fuller

Mason Howe

Michael Caniglia

Andrea Holgado

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Generating *Caenorhabditis elegans* UNC-33 antigens to be used for the Synthesis of Polyclonal Antibodies

by Matt Abbott, Jacob Fuller, Mason Howe, Michael Caniglia
(Prof. Andrea Holgado, Department of Biological Sciences)

UNC-33 and its human homolog, CRMP2 (Collapsin Response Mediator Protein-2), have been demonstrated to be involved in neurodevelopment as well as neurodegenerative disorders, primarily Alzheimer's Disease. However, the physiology and interactions of these associations are vague. In order to further understand UNC-33/CRMP2, our group decided to use molecular biology and work toward the production of polyclonal antibodies specific to *C. elegans* UNC-33. To do this, we utilized the GST tag Gene Fusion System and produced two antigens- UNC-33 amino acid 48 to 212 and UNC-33 amino acid 48 to131 (UNC-$33_{48\text{-}212}$ and UNC-$33_{48\text{-}131}$). During this process, parameters were developed for the efficient expression and purification of these polypeptides. Once an effective protocol was established, GST fused UNC-$33_{48\text{-}212}$ and UNC-$33_{48\text{-}131}$ were expressed, purified, and tested for purity multiple times. Overall, these procedures resulted in the production of 3.72 mg and 2.10 mg of GST fused to UNC-$33_{48\text{-}212}$ and GST fused to UNC-$33_{48\text{-}131}$, respectively. Currently, these purified polypeptides are being injected into laboratory animals for the generation of polyclonal antibodies for UNC-33 research.

Introduction

UNC-33/CRMP is a family of cytosolic proteins conserved from worms to humans. In the nematode *Caenorhabditis elegans (C. elegans)*, the *unc-33* gene encodes for three splicing isoforms, UNC-33L (long), UNC-33M (medium), and UNC-33S (short)

(1,3-5). Studies of these protein isoforms demonstrate that UNC-33 proteins play a vital role in neuronal development and elongation of axons. Molecular analyses of these functions showed that UNC-33 proteins modulate the organization of actin filaments and microtubules within the axonal growth cone (1,2). Conversely, CRMP2, the vertebrate homolog of UNC-33, is heavily involved in promoting axonal specifications and differentiation (3,6-11). Within its normal bounds, CRMP2 binds tubulin to stabilize microtubules and facilitates protein trafficking (7,12-13). However, analyses of autopsies of patients suffering Alzheimer's Disease (AD) showed that CRMP2 is heavily represented in toxic intracellular protein aggregates (7,12-13). For instance, examinations of the cortex, hippocampus, and isocortex identified that hyperphosphorylated CRMP2 accumulates at neurofibrillary tangles (NFTs) and β-amyloid (Aβ) peptide-rich plaques of AD patients (9,12,15-16). Furthermore, studies using a mouse animal model for AD suggested that rescue of CRMP2-dependent mechanisms play a role in the prevention of synapse loss and neurodegeneration (12). Consequently, Hensley and others postulated that CRMP2 could be an important target molecule for the development of experimental neurodegenerative disease therapeutics (9,17-20). Since then, studies from various groups demonstrated that lanthionine ketimine ethyl ester (LKE) binds to CRMP2 and rescues neuronal circuitry defects associated with mutations of the *unc-33* gene (22, 23, 24).

Thus, to further investigate UNC-33 isoforms and ascertain the molecular bases of LKE action, we began producing two UNC-33 antigens for the production of polyclonal antibodies. To the present time, Kaibuchi and colleagues reported utilizing polyclonal antibodies raised against UNC-33-glutathione s-transferase (GST) fusion protein. This study also established the existence of three splicing forms of UNC-33 – UNC-33L, UNC-33M, and UNC-33S, based on three alternative start codons and a common stop codon (1).

Herein, we present strategies followed for the production of two truncated UNC-33 fusion proteins and their purification.

Truncated UNC-33 fusion proteins were made using two plasmids, the first plasmid coding for UNC-33 amino acids 48-212 was produced envisioning the production of an antibody that could detect isoforms UNC-33L and UNC-33M. The second plasmid coding for UNC-33 amino acids 48-131 was to be used for the generation of antibodies detecting of UNC-33L alone (1). Several parameters were investigated during the process of manufacturing these antigens, including the length of isopropyl-beta-D-thiogalactopyranoside (IPTG) induction time, troubleshooting the GST fusion affinity purification, filtration and dialysis of the GST fused UNC-33 proteins, and subsequent preparation for polyclonal antibody production. The proper concentration of glutathione sepharose beads and glutathione solution to be utilized was also established. In preparing for polyclonal antibody production, the protein of interest was concentrated and the elution product dialyzed vs. saline solution. This research project resulted in the production of 3.72 and 2.10 total mg of GST fused UNC-$33_{48\text{-}212}$ and UNC-$33_{48\text{-}131}$, respectively.

Materials and Methods

GST fusion expression plasmids and bacterial transformation

Plasmids ah139 (coding for GST fused UNC-$33_{48\text{-}212}$) and ah142 (coding for GST fused UNC-$33_{48\text{-}131}$) were kindly produced by LaKesha Seals using conventional molecular biology techniques, restriction enzymes *Sal* I and *Bam H*I, UNC-33 cDNA coding for amino acid 48-212 and 48-131, and pGEX 4T1 plasmid (GE Healthcare Life Sciences). Plasmids ah139 and ah142 were transformed into BL21-Gold (de3) pLys 5 competent cells (Stratagene) using the manufacturer recommendations.

UNC-33 Protein Induction Times

Overnight liquid cultures of transformed BL21-Gold (de3) pLys 5 cells were diluted 1/50 using 2xyT broth containing 100 µg/mL ampicillin, and incubated in a shaker at 250 rpm, 37°C for 1-4 hours. During that span of four hours, the cultures were checked every hour until they reached an Optical Density$_{600}$ (OD_{600}) of 0.6-0.8. Once desired OD_{600} was reached, IPTG at 0.5mM of the

final concentration was added to each culture to induce protein expression. Cultures were allowed to continue growing in a shaker at 250 rpm, 37°C until overnight. Sample culture aliquots were taken before induction with IPTG, every two hours from 0-8 hours after induction, and overnight. Cultured cells harvested from each sample were centrifuged at 15,700 x g for 1 minute and their pellets were prepared for SDS-PAGE analysis using 1X XT sample buffer (BioRad) and 1 X reducing agent (BioRad). Last, samples were heated at 100°C for 5 minutes, and run for 1 hour at 150 V using precast Criterion gels (4-12% XT MES)(BioRad) and manufacturer recommendations.

Purification of GST Fusion Proteins

Cultures grown and induced according to the optimal empirical time were centrifuged at 5000 x g at 4°C for 10 minutes. The supernatant was decanted and the cells were stored at -80°C.

Next, cells were resuspended in 1X PBS solution containing 1X EDTA and 1X protease inhibitor (ThemoScientific). The resuspended pellets were transferred into 50 mL conical tubes and sonicated for 3, 30-second intervals with 30 seconds of incubation on ice in between. Triton x-100 in PBS was added to a final concentration of 1% and mixture was then incubated on a rotary shaker for 20 minutes at 4°C. Immediately following the incubation period, the lysate was centrifuged for 20 minutes at 3,220 x g, 4°C. Tubes containing 2 mL of glutathione sepharose beads per 500 mL of original bacterial culture were washed with 1X PBS and centrifuged for 1 minute at 3,220 x g, 4°C. This wash process was repeated a total of three times. Once the centrifugation of the lysate was completed, the supernatant was divided evenly between the two tubes containing the beads and incubated on a rotary shaker overnight at 4°C. A 50 µL aliquot was taken of the supernatant before mixing with the beads (crude extract), and prepared for SDS-PAGE analysis following the procedure mentioned previously.

Cell lysate was incubated overnight with the GST purification resin to allow desired proteins to bind to sepharose beads. Samples were centrifuged for 30 seconds at 3,220 x g, 4°C, and before the supernatant was decanted and discarded, a 50 µL aliquot (unbound)

was taken for SDS-PAGE analysis. Bead pellets containing purified protein were washed 3 times with10 mL of PBS each time. Last, GST fused UNC-33 proteins were eluted from beads using freshly made 2 mL of 15 mM glutathione solution and incubating on rotary shaker overnight at 4°C. After elution, the samples were centrifuged for 30 seconds at 3,220 x g, 4°C, and the supernatant was transferred to a clean tube for storage. Beads were washed and reconstituted for future purifications. A sample aliquot of the eluted (supernatant) and non-eluted (beads) was taken SDS-PAGE analysis.

SDS-PAGE analysis

Samples prepared for SDS-PAGE analysis were run for 1 hour at 150 V using precast Criterion 4-12% XT MES gradient gels (BioRad) and manufacturer recommendations. Proteins on gel were stained with Coomassie brilliant blue R-250 (BioRad) for 1 hour and destained overnight. Stained gels were imaged using the ChemiDoc imaging system (BioRad)

Concentration via Amicon® Centrifugal Filter Device

Eluted purified GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ were concentrated using the Amicon® Ultra-15 10K Centrifugal Filter Devices (Millipore) and manufacturer recommendations. In brief, using Amicon®'s Typical Spin Time to Filtrate Volume Profile, the samples were centrifuged at 3220 x g and 1-minute intervals, checking after each centrifugation until the desired volume remained (27).

Protein Dialysis

Affinity purified antigens were dialyzed using a Slide-A-Lyzer Dialysis Cassettes (10K MWCO) (ThermoScientific). Solution containing proteins of interest were injected into the dialysis cassette following the provided protocol (20). Dialysis cassettes were covered with 1X PBS and incubated by stirring overnight at 4°C. The dialyzed protein solution was removed by following the provided protocol as well (26). The purified protein was stored at -20°C.

Determination of Protein Concentration

Protein concentration was determined by quantifying the

density of Coomassie brilliant blue R-250 stain in affinity purified protein samples and BSA (Bovine Serum Albumin) standards ranging from 80-1000 ng. Standards and other protein samples run on the same gel were imaged using image lab software (Bio-Rad). Volume tools menu of the image lab software (Bio-Rad) was utilized to produce a standard curve while concentration of unknowns was automatically determined from the line of best fit.

Results

GST::UNC-33 Induction Times

As demonstrated previously, the UNC-33 protein family consists of three different splicing forms UNC-33L, UNC-33M, UNC-33S (1). In order to pursue an understanding of the functional roles and expression patters of these three different splicing forms, we began the process of producing antigens GST::UNC-$33_{48\text{-}212}$ for the production of antibodies detecting UNC-33L and UNC-33M, and GST::UNC-$33_{48\text{-}131}$ for the detection of UNC-33L only. To do so, determining the optimum induction time for the expression of these proteins was the first of many steps leading to the synthesis of UNC-33 antigens. First, plasmids ah139 and ah142 were introduced into BL21-Gold (de3) pLys 5 cells, and the optimal time of induction leading to the ideal amount of GST::UNC-33 was determined empirically. Samples collected of various time periods after IPTG-mediated induction were analyzed using SDS-PAGE. Results shown in figure 1 revealed that GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ are being expressed at quantifiable amounts and poses a predicted size of 50 kDa and 37 kDa, which match calculations derived from the amino acid composition. Moreover, analysis of induction times for both GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ showed that the intensities of bands observed after 4-6 hours of induction are about the highest. At eight hours of induction, the bands either disappeared or lessened in signaling, suggesting a lower concentration of the proteins of interest.

(See figure 1 on page 38)

Affinity Purification of GST::UNC-33 antigens

Purifying GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ was vital in

the production of polyclonal antibodies for splicing isoforms. Once we determined the optimum expression protocol, we continued with the adaptation of a affinity purification procedure that best fit that of GST::UNC-33. Utilizing the GST tag, affinity purification was achieved using the glutathione sepharose beads and glutathione solution. Beads containing glutathione bind to GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ with high affinity. Glutathione solution, on the other hand, elute affinity purified GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ by competing for binding and displacing GST fusion proteins from beads. As shown in figure 2, we successfully purified both UNC-33 antigens from crude cell extracts and eluted most of the GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ from beads.

(see figure 2 on page 38)

Concentrating UNC-33 antigens

Since results from 4 batches of protein affinity purification for GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ produced diluted samples of 0.202 mg/mL and 0.255 mg/mL, respectively. Fusion proteins were concentrated to an approximate target of 1.0 mg/mL. Concentration of affinity purified proteins was accomplished using the Amicon® Ultra-15 10K Centrifugal Filtration Device and centrifugal forces. Results from this procedure produced a volume reduction of 12mL to 2.5 mL in the case of GST::UNC-$33_{48\text{-}212}$ and 12mL to 3 mL for GST::UNC-$33_{48\text{-}131}$mL after eight minutes centrifugation at 3220 x g.

Dialysis and Determination of Protein Concentration.

After purification and concentration of proteins of interest, dialysis was necessary to remove glutathione from the elution sample. Dialysis against 1X PBS was performed using the Thermo Scientific Slide-A-Lyzer® Dialysis Cassettes, which permit the diffusion of substances smaller than 10 kDa. Once dialysis was complete, total protein content and concentration were determined by densitometry of SDS-PAGE gels. Intensity of Coomassie brilliant blue R-250 stained bands was quantified and GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ were compared to BSA Standards (Figure 3 and Figure 4). Results from this measurement led to the calculated concentrations equaling 1.24 mg/mL and 0.84 mg/mL for

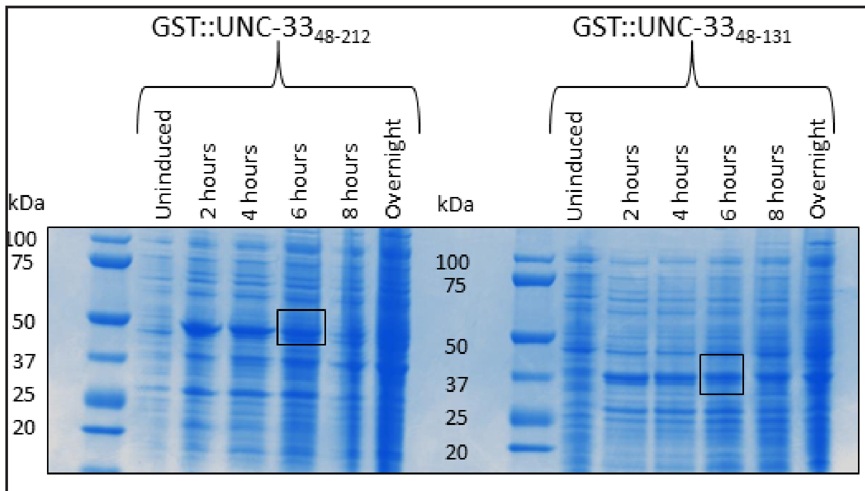

Figure 1. The optimal induction time for both GST::UNC-33$_{48-212}$ and GST::UNC-33$_{48-131}$ was determined to be around 4-6 hours at 37°C. Samples of BL21-Gold (de3) pLys 5 cultures expressing proteins of interest were analyzed via SDS-PAGE. Images obtained after Coomassie brilliant blue R-250 staining demonstrated that induction produced the highest amount of protein at times 4-6 hours and these proteins match the expected sizes of 50 and 37 kDa, respectively.

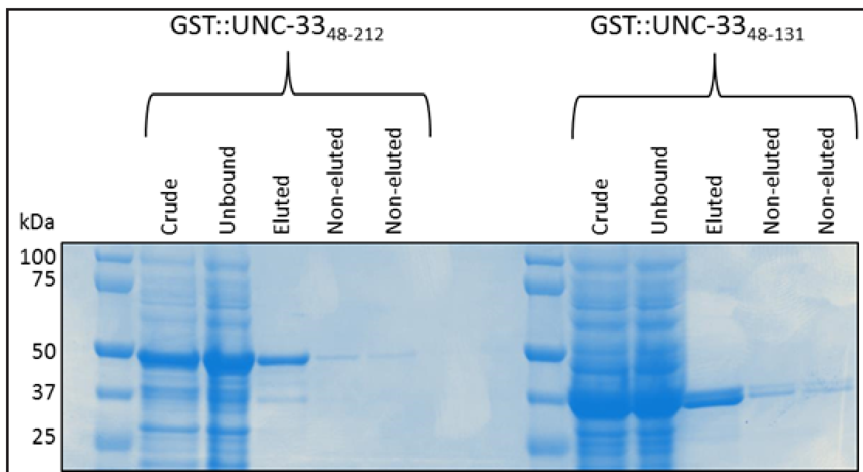

Figure 2. GST fusion affinity purification protocol successfully produces antigens GST::UNC-33$_{48-212}$ and GST::UNC-33$_{48-131}$. Analysis of various samples of the affinity protein purification process indicates that GST::UNC-33 is purified from crude cell extracts and eluted from beads using a glutathione solution. Samples examined were crude cell extract (crude), unbound fraction (unbound), eluted and two tubes containing beads (non-eluted).

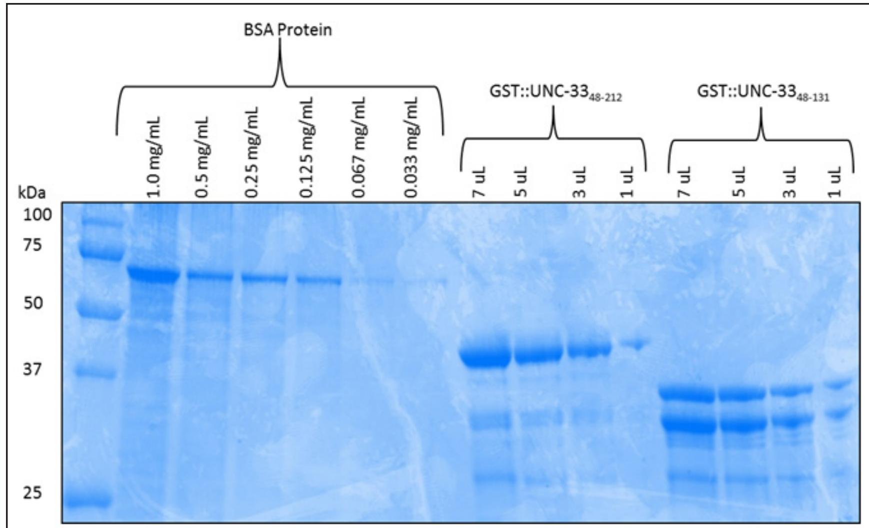

Figure 3. The comparison of GST::UNC-33$_{48\text{-}212}$ and GST::UNC-33$_{48\text{-}131}$ to BSA Standards of different concentrations to determine the concentration of the proteins of interest.

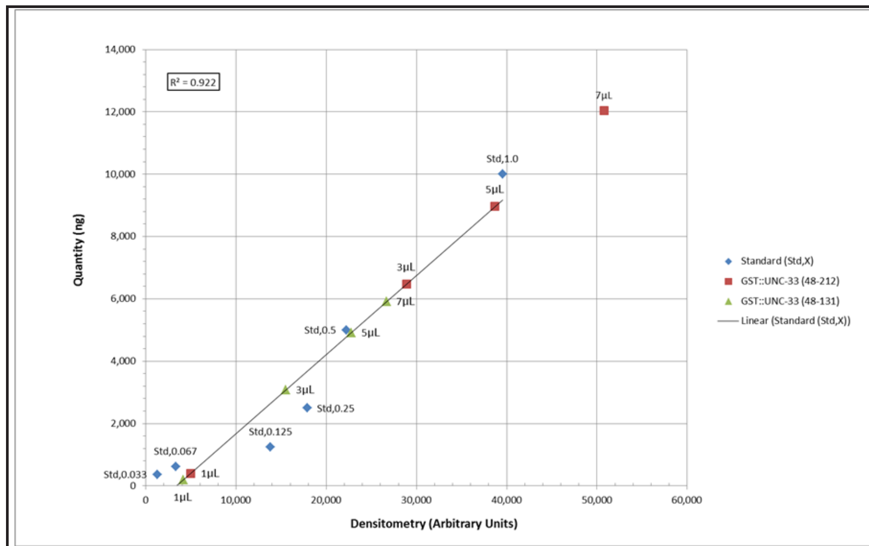

Figure 4. Standard Curve of BSA Standards of known concentration used to determine the concentration of GST::UNC-33$_{48\text{-}212}$ and GST::UNC-33$_{48\text{-}131}$. Sample indicators match those shown in Figure 3.

GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$, respectively.
(see figures 3 and 4 on page 39)

Discussion

Kaibuchi and colleagues were the first group studying UNC-33 in *C. elegans* and producing antiserum samples raised against amino acids 1-121 of UNC-33L (antibody anti-UNC-33L) and full-length UNC-33S (antibody anti all UNC-33 isoforms) (1). Using these antibodies, the group characterized the expression pattern of endogenous UNC-33 isoforms in *C. elegans* as well as the nature of the mutation in *unc-33* mutant alleles. This investigation exhausted all the anti-serum containing UNC-33 polyclonal antibodies. Therefore, continuation of research centered on UNC-33 isoforms required the redevelopment of molecular tools that in this case will recognize one, two, or potentially all three isoforms. To fill this gap and further examine UNC-33 isoform expression pattern throughout development, *in situ* localization, and protein-protein interactions, we presented herein initial steps towards the production of antibodies specific for *C. elegans* UNC-33 isoforms. Even though a conventional system for the production of GST fusion proteins was employed, the steps leading to manufacturing the antibodies for UNC-33 required adapting an affinity purification protocol for the successful production of GST::UNC-33 proteins. Troubleshooting this procedure has required tedious attention as the basis for the creation of GST::UNC-33 was performed for the first time in our laboratory and preceding published information on these terms is not fully disclosed. First, determination of proper induction time was crucial, since induction times that are too long or not long enough can detrimentally affect the efficiency of the purification and amount of protein produced. Upon adding IPTG to the transformed cells, optimal production of the protein of interest took about 4-6 hours (Figure 1). This result is in agreement with previous work showing that the 4 to 6 hours after the addition of IPTG is sufficient for the displacement of the LacI repressor and induction of the Lac promoter (25). Thus, production of the two variants of GST::UNC-33 was maintained at the six hour time

induction period.

In regard to the affinity purification of GST::UNC-33, the protocol adapted led to qualitatively and quantitatively sound results, but not without the need for improvement. Based on Figure 2, both GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ were present in the unbound and non-eluted samples. The unbound sample corresponds to the post-binding supernatant that contains proteins, which failed to bind to glutathione sepharose beads. The non-eluted sample comprises the glutathione sepharose beads after treatment with soluble glutathione. To decrease the percentage of unbound and noneluted proteins, we increased the amount of beads used for the purification and extended the time of elution. These changes led to an increased yield of the protein of interest. However, additional degradation products were observed. The appearance of these degradation products were resistant to protease inhibitors and EDTA (Figure 3).

After several batches of affinity purification procedures, diluted samples of GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ were concentrated with the Amicon® Ultra-15 Centrifugal Filter Device, which was reported to be effective in retaining proteins larger than 10 kDa. The protocol indicated by Amicon® led to the prediction that centrifugation at 4,000 x g for approximately seven minutes should filtrate a 10 mL solution (27). Empirical results obtained by our group show that eight minutes were sufficient for filtrating between 9.0 and 10.0 mL of solvent.

The tripeptide Glutathione used during elution can result in undesired effect when injected together with the antigen. To remove and exchange the glutathione with PBS, we dialyzed the concentrated protein sample with Slide-A-Lyzer® Dialysis (26). This technique proved to be effective in exchanging the solvents and further removing contaminants from the protein samples.

Together, this research project resulted in the generation of 3.72 and 2.10 mg of GST::UNC-$33_{48\text{-}212}$ and GST::UNC-$33_{48\text{-}131}$ at concentrations of 1.24 mg/mL and 0.84 mg/mL respectively. Although the ultimate step in this investigation is antibody manufacturing, its success is critically dependent on the production

of quality GST::UNC033 antigens. Moreover, the protein products produced during this research project have the potential to transform our current understanding of UNC-33/CRMP2 in health and disease.

Acknowledgements

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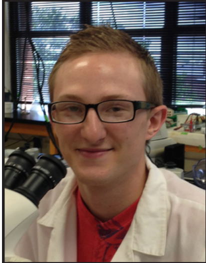
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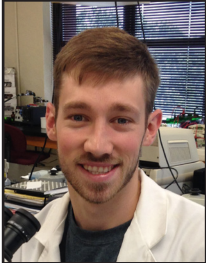
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