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Transformation of Model Organisms with Munc13-1 for Subcloning and Protein Harvesting

Grace Dittmar

The University of Southern Mississippi

Transformation of Model Organisms with Munc13-1 for Subcloning and Protein Harvesting

by

Grace Dittmar

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Approved by:

_____

Hao Xu, Ph.D., Thesis Adviser
Professor

_____

Jake Schaefer, Ph. D., Director School of Biological, Environmental, and Earth Sciences

_____

Ellen Weinauer, Ph.D., Dean
Honors College

Abstract

Exocytosis is the process by which cells release various molecules into the extracellular space. This mechanism is accomplished by membrane fusion, in which the membrane of the transport vesicle and that of the cell itself fuse to create one continuous membrane, allowing the release of the molecules inside the vesicle. A family of proteins, known as SNARE proteins, is responsible for facilitating membrane fusion. SNARE proteins form a complex between the vesicle membrane and the plasma membrane. For various cells, such as mast cells and neurons, exocytosis is essential to carry out their vital functions. Various accessory proteins are known to play a role in membrane fusion by regulating the assembly and disassembly of the SNARE complex. One family of proteins is known as Munc13 proteins. This protein family has been shown to regulate SNARE complex formation both positively and negatively in mast cells and neurons. While three isoforms are cell-specific, Munc13-1 has been found to regulate degranulation in both neurons and mast cells. In mast cells, Munc13-1 has been found to inhibit the activity of degranulation stimulating protein Munc13-4, and to decrease the level of antigen-induced degranulation experienced by the cell. However, in neurons, Munc13-1 helps to convert syntaxin from the closed Munc18-syntaxin formation to the open SNARE complex formation. This project aims to transform two expression systems with a truncated version of Munc13-1 gene from two different plasmids to use in further studies.

Keywords: Mast Cell, Degranulation, Munc13, Neuron, SNARE-Complex

Dedication

I dedicate my thesis to my biggest fan, greatest role model, and best friend: my grandfather.

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First, I would like to thank my thesis advisor, Dr. Hao Xu, for his consistent support throughout the past two years I have worked in his lab. Dr. Xu, you have taught me so much about what it means not only to perform high quality research, but to be a high quality researcher myself. Thank you for always taking the time to fully answer every question I had, no matter how small.

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List of Abbreviations

Dunc13-Drosophelia uncoordinated 13

FC - Fragment crystallizable

IgE- Immunoglobulin E

Munc13-Mammalian uncoordinated13

RBL- Rat Basophilic Leukemia

SNARE-Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

VAMP-Vesicle associated membrane protein

Chapter 1: Introduction

Cells increase their ability to survive by maintaining a stable state of equilibrium between themselves and their environment, called homeostasis. One essential function for cells to maintain homeostasis is the ability to regulate what enters and exits the cellular membrane, which is regulated by membrane fusion (14). The process of membrane fusion involves the uniting of two individual membranes as one seamless bilayer and is mediated by various proteins and complexes (14).

A family of proteins known as Soluble N-ethylmaleimide-sensitive factor attachment protein receptors proteins (SNARE proteins) is known to catalyze the process of membrane fusion (9). Three proteins that dominate this complex are Syntaxin, Snap 25, and synaptobrevin/ vesicle-associated membrane protein (VAMP). These three proteins are then further categorized as either V-SNARES or T-SNARES (9). Syntaxin and SNAP 25-like protein are known as T-SNARES, due to their placement on the target membrane of the cell. Synaptobrevin is known as a V-SNARE because it is located on the actual transport vesicle itself. The SNARE complex is formed upon the interaction of T-SNARES and V-SNARES from two different membranes (9).

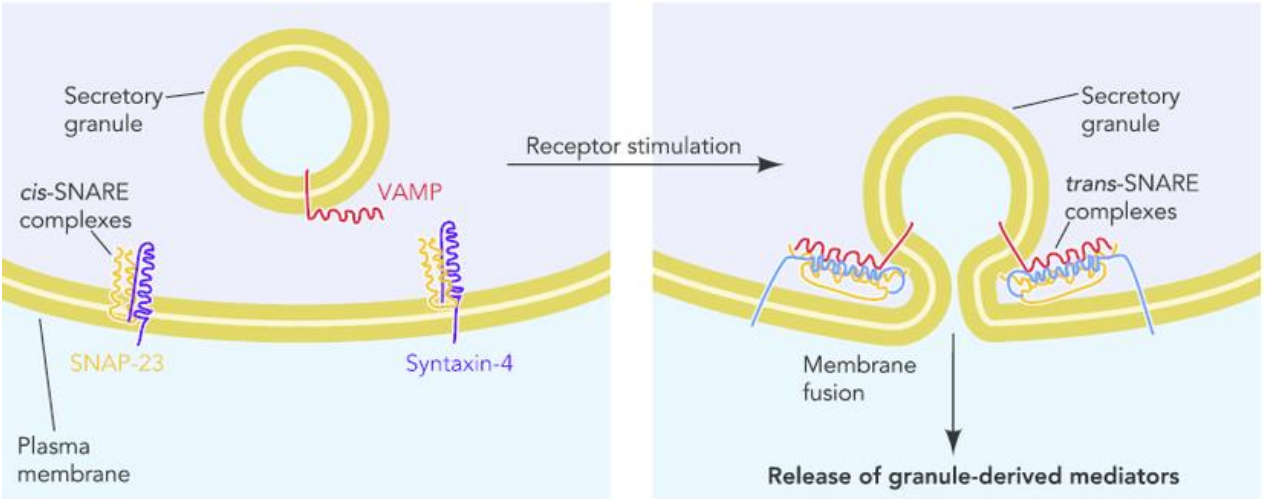


Figure 1. An external stimulus activates a mast cell. The V-SNARE complexes on the secretory granule form a trans-SNARE complex with the cis-SNARE complexes (comprising of SNAP 25 protein and Syntaxin). This trans-SNARE complex allows for the fusion of the two membranes and the release of the granule contents.

The formation of this complex causes stress to the lipid bilayer and allows for the two membranes to fuse and release the transport vesicles contents (Figure 1). The specific mechanism by which cells undergo membrane fusion is not fully understood. The process is known to be complex, involving a variety of different proteins and modulators (10). Transport vesicles, such as granules, are packed and processed in the Golgi Apparatus and sent through various transport carriers to the plasma membrane of the cell (10). Membrane fusion then occurs between the secretory vesicle membrane and that of the cell itself which leads to release the contents of the vesicle into the extracellular space (10).

Membrane fusion is a vital part of exocytosis in cells, especially in effector cells such as mast cells. Degranulation is the name given to a specific form of exocytosis in mast cells, in which they release the contents of their granules (11). As an effector cell, mast cells sense changes in environmental stimuli and respond appropriately. Mast cells are a critical component of the immune system, and their secretory granules are an essential component of their immune responses (11). A mast cell protects the body against damage from potential pathogens, stress, or antigens (11). An antigen is any substance capable of warranting an immune response, such as one carried out by a mast cell. Once an antigen is detected, mast cells are said to be "activated," and degranulation occurs approximately 5-10 seconds afterward (11). In degranulation, bioactive molecules, such as histamines, chemokines, or proteases, stored in secretory granules are released from the cell to combat extracellular threats (11).

Both the adaptive and innate immune systems utilize mast cells. However, mast cells are most commonly associated with allergic reactions and are stimulated by IgE (Immunoglobulin E) antibodies (15). This Type 1 Hypersensitivity reaction occurs when an antigen binds an antibody, forming an antigen-antibody complex (15). This complex contains a fragment crystallizable (FC) region, which binds to an FC receptor on the exterior of the mast cell (15). The binding of the FC receptor on the mast cell to the FC region on the antigen-antibody complex activates the mast cell, which undergoes degranulation and releases a variety of mediators that will carry out an immune response (Figure 2).

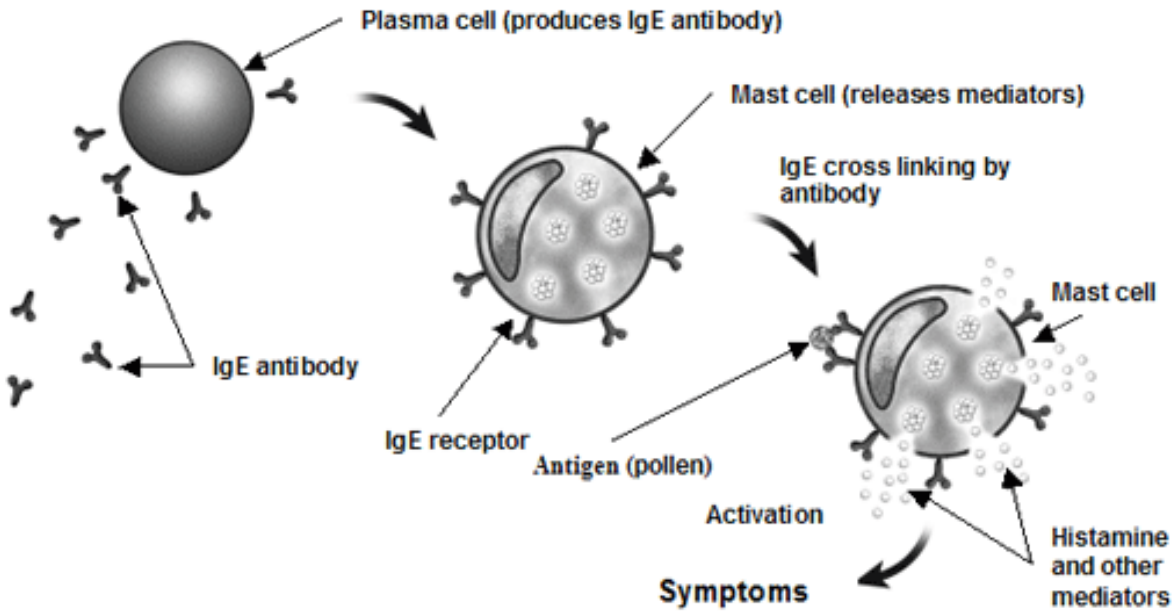


Figure 2. Antibodies made by plasma cells bind antigens, forming antigen-antibody complexes. An antigen-antibody complex then binds specific receptors on the surface of a mast cell and activates the cell. The activated mast cell then releases effector molecules, and a cellular response occurs.

Although the mechanism of membrane fusion appears to be highly conserved in all eukaryotic organisms, the regulation of this process varies. The regulation of mast cell degranulation complex is important because unregulated or miss-regulated degranulation can lead to hyper-inflammation and tissue damage. Initially, studies to curb destructive mast cell degranulation focused on preventing the formation of the antigen-antibody complex with IgE. However, recent findings have pointed towards the regulation of the assembly and disassembly of the SNARE complex as a possible source of therapeutic treatment. For this reason, the accessory proteins that help regulate mast cell degranulation are of great interest.

One family of proteins that is associated with the regulation of SNARE mediated membrane fusion are the Mammalian uncoordinated13 proteins (Munc13 proteins). A defining feature of Munc13 proteins is their various domains, such as C2 domains, phospholipid binding domains, and calcium binding domains, which interact directly with SNARE proteins (3). Munc13-4 is an isoform of the Munc13 protein that is highly associated with mast cell degranulation and is theorized to play a role in vesicle priming (3). Support for this theory comes from a 2018 study that showed rat mast cells with a deleterious mutation in Munc13-4 were able to dock secretory vesicles, but unable to perform exocytosis fully (3). The terminal end of the C2A domain Munc13-4 has been identified to participate in priming by stabilizing the secretory granule and restricting its movement abilities both in the binding of Rab27a and at the plasma membrane (5). However, another isoform has been found to play a role in the negative regulation of degranulation. Recent studies have found evidence that Munc13-1 may inhibit degranulation in mast cells via suppression of Munc13-4 (6). Experiments using mast cells with Munc13-1 knockdown showed a significant increase in antigen-induced degranulation in mast cells (6). Likewise, it was found that the overexpression of Munc13-1 led to a significant reduction in

antigen-induced degranulation. In addition to the decrease in degranulation, the overexpression of Munc13-1 led to a decrease in the expression of Munc13-4, thus suggesting an inhibitory effect on the production of the protein (6).

Mast cells, however, are not the only cell in which exocytosis is a vital function. Neurons are cells that play a fundamental role in the nervous system by performing exocytosis to release neurotransmitters (16). Like other cells neurons receive signals from the environment and respond accordingly (16). However, these highly specified cells are different from mast cells by the way in which they carry out a response. Neurons work by sending electrical messages to one another using action potential as a method to power the signal, a term known as neuron "firing" (16). First, much like any other cellular response, there must be an environmental shift the neuron becomes aware of. In the case of neurons, this signal is the disruption of normal concentrations levels in the cells membrane (13). The altering of concentrations leads to depolarization, followed by repolarization which results in the generation of action potential (13). The action potential propagates down the axon to where it reaches the axon terminal. Upon reaching the axon terminal, an influx of Ca2+ rushes into the cell, and allows the membrane of the synaptic vesicle to fuse with that of the neuron membrane, thus releasing the contents of the vesicle into the synapse (Figure 3).

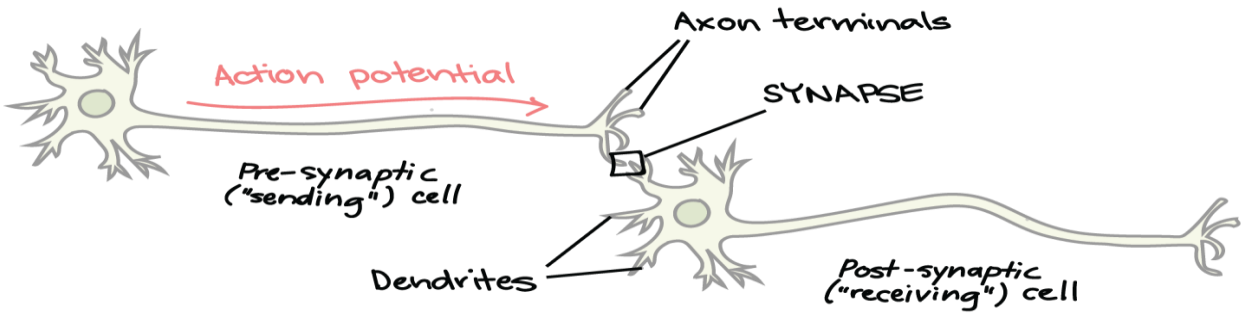


Figure 3. Action potential travels from the soma (cell body) of the neuron down the axon. Once it reaches the axon terminal, neurotransmitters are released into the synapse where the chemical signal will be received by another neuron.

A fundamental difference between mast cell degranulation and that of the release of neurotransmitters by neurons is the "green light" in which they receive to perform exocytosis. Mast cells are activated by the formation and binding of an antibody-antigen complex, as previously discussed. Neurons, on the other hand, are only able to undergo degranulation once an action potential reaches the axon terminus (13). However, neurotransmitter release in neurons is very similar to degranulation in mast cells, such as the mechanism of SNARE complex formation, using the same T-SNARES and V-SNARES (Figure 4). Likewise, proteins from the Munc13 family are also heavily involved in the regulation of the SNARE complex formation in neurotransmitter release (16).

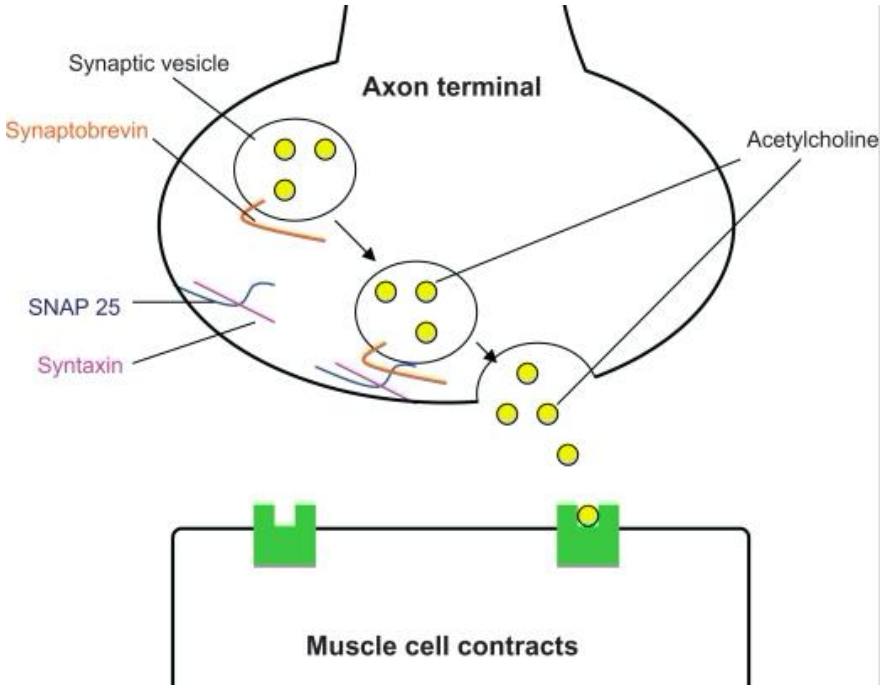


Figure 4. After an action potential reaches the axon terminal, the T-SNARES on the transport vesicle interact with the V-SNARE on the neuron's membrane, forming the SNARE-complex. The formation of this complex leads to the release of a neurotransmitter, in this case,

Though previously mentioned to have inhibiting roles in mast cell degranulation, most functions of Munc13-1 have been identified in neurons, along with Munc13-2 and Munc13-3. However, Munc13-1 is by far the isoform with the highest amount of expression in the brain of all the Munc13 isoforms and is believed to participate also in priming of the secretory vesicle (4). The Munc13-1 isoform is expressed in the olfactory region, the cerebellum, the cortex and the hippocampus (4). Similar to studies on Munc13-4, studies on mice without Munc13-1 showed a severe limitation in neurotransmitter release (1). Additionally, elimination of this isoform in both *Drosophila* uncoordinated 13 (Dunc13-1) and in *C. elegans* (Unc13-1) yielded similar results (2 & 12). The isoform Munc13-1 is thought to regulate the priming of a vesicle by directly interacting with the N-terminal region of Syntaxin (T-SNARE) with a coiled domain of its own (7). Further studies in mice showed that mice without Munc13-1 or Munc13-2 were completely unable to release neurotransmitters, and demonstrated severe difficulty with docking secretory vesicles (4).

Munc13-1 has several active domains which are believed to work alongside an additional accessory protein, called Munc18, to facilitate membrane fusion (8) (Figure 5).

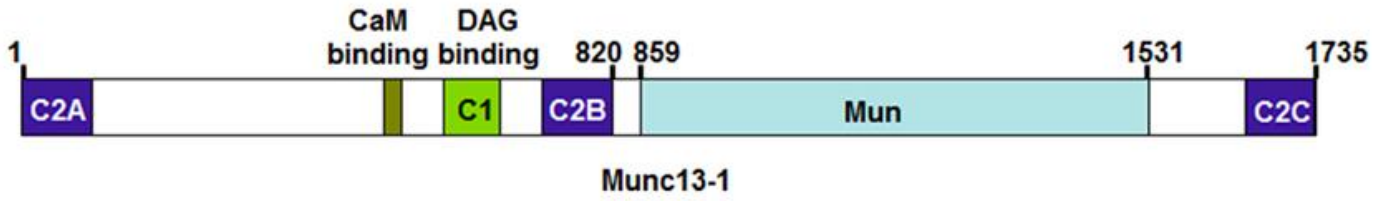


Figure 5. Munc13-1 gene with three C2 domains, a C1 domain, and a MUN domain.

Munc18-1 binds Syntaxin to form a Munc18-1/Syntaxin complex, that keeps Syntaxin in its closed formation (7). This action prevents the SNARE complex from reassembling. The MUN domain in Munc13-1 catalyzes the movement of Syntaxin from the closed Munc18-1-Syntaxin complex to the SNARE complex (7). Additionally, the MUN domain of Munc13-1 has also been found to assist in the proper configuration of the Syntaxin/ SNAP-25/ Synaptobrevin complex from the Syntaxin/SNAP-25 complex and the separate Synaptobrevin through interaction with the cytoplasmic domain of Syntaxin (7). It has also been discovered that Munc13-1 promotes the proper formation of the Syntaxin/SNAP-25 itself, though the specific mechanism is not yet known. Domains C1 and C2B are also believed to play vital role in fusion, as when isoforms without these regions were tested they displayed significantly lower levels of membrane fusion (7).

As discussed, Munc13-1 has regulation properties in the membrane fusion of both mast and neuron cells. In mast cells, Munc13-1 helps to combat over degranulation which may lead to rapid inflammation and tissue damage (15). In neurons, it aids in the priming of the secretory vesicle and thus the success of releasing neurotransmitters (7). Inability to release or the over release of neurotransmitters can lead to an abundance of medical issues that can be potentially life-threatening (7). For these reasons stated, it is clear to see that Munc13-1 is vital to the success of an organism. Thus, the study of this isoform is critical in further understanding of membrane fusion defects in both mast cells and neurons. The availability of a purified Munc13-1 protein readily available is possible through the transformation of the gene into a cloning plasmid and the transformation of that plasmid into a model organism for protein synthesis. In this experiment we aim to transform two expression systems with truncated Munc13-1 to harvest purified versions of the protein for further studies.

Chapter 2: Methods and Materials

2.1 Plasmid Information

We received wo plasmids from UT Southwestern Medical Center, one being pFastBac GST C1C2BMunC2C, and the other pET28a C1C2BMunC2C. Both constructs were previously truncated to only include the amino acids 529-1735, with the amino acids 1408-1452 being replaced with an EF sequence.

2.2 Transformation into DH5 Alpha Cells

Competent cells were removed from the -80 °C freezer that they are stored in and allowed to thaw on ice for 5 minutes. In two separate 1.5mL microfuge tube, 100uL of competent cells were added along with 10uL of either pFastBac GST C1C2BMunC2C or pET28a C1C2BMunC2C. The tubes were mixed by gently flicking the sides and then allowed to incubate on ice for approximately 30 minutes. After the conclusion of the incubation period, the cells were heat shocked at 42°C for exactly 45 seconds using a heat block. After heat shocking, the cells were immediately returned to ice to incubate for an additional 10 minutes. Then, 75uL of SOC media was added to each of the two tubes, and the cultures were incubated for 60 minutes at 37°C.

2.3 Preparation of LB Plates with Antibiotics

LB plates with selective media were prepared to select for transformed cells. 10g of Bactoagar was measured out in a fresh weigh boat. 5g of Bactoagar was then transferred to two sterilized 500mL bottle. 600mL of LB media was measured using a 1000mL sterilized cylinder. 300mL of liquid LB media was then transferred to each 500mL bottle. The bottles were labeled appropriately and autoclaved for 20 minutes on the liquid cycle. After sterilization by autoclave, 300µL of Ampicillin (100µg/mL) was added to one bottle and 300µL of Kanamycin (50µg/mL)

was added to the other. The bottles were then mixed. 20mL of the mixture was poured onto each empty petri dish next to a Bunsen burner to prevent contamination. Plates were labeled, allowed to solidify, and stored at 4 °C.

2.4 Plating of Cells from Bacterial Stock

After an incubation period of 60 minutes at 37°C, 150µL of pFastBac GST C1C2BMunC2C mixture was plated on LB/ Ampicillin (100µg/ml) [PLATE A] and 150µL pET28a C1C2BMunC2C mixture was plated on LB/Kanamycin (50µg/mL) [PLATE B]. 5-7 sterile glass beads were added to each plate, and the cells were spread by gently rotating the plates in a circular motion to spread the beads. Plates were then placed upright in an incubator at 37°C for 20 minutes. After 20 minutes, the plates were inverted and allowed to incubate overnight.

2.5 Inoculation of Culture

The workbench was sterilized with 70% ethanol and a Bunsen burner was turned on using a strike lighter. Using a pipette aid, 6mL of sterile LB media was transferred into two pre-autoclaved test tubes. The test tubes were labeled for either pFastBac GST C1C2BMunC2C [TUBE 1] or pET28a C1C2BMunC2C [TUBE 2]. 6µL of Ampicillin was then added into TUBE 1, while 6µL of Kanamycin was added into TUBE 2 Using the pipette aid, the media was mixed thoroughly by pipetting up and down several times. Then using two wooden sticks that were sterilized, a loop of bacteria from PLATE A was added to the TUBE 1, and a loop full of bacteria from PLATE B was added to TUBE 2. When transferring the bacteria, it was noted that each loop full came from one single colony on the plate and that the wooden stick did not touch the sides of either tube during transfer. The tubes were then mixed well by brief vortexting and stored in a shaking incubator overnight at 37°C.

2.6 Plasmid Isolation

After an incubation period of 24 hours, the two tubes were removed from the shaking incubator and placed on ice. Four sterilized microfuge tubes were obtained and labeled, two for HXB-OJ41 [TUBE 1] and two for HXB-OJ39 [TUBE 2]. For each plasmid, samples were designated sample A and sample B. Using a pipette aid 1.5mL of the culture was transferred into each appropriate tube, and centrifuged at 13krpm for 1 minute at 4°C. The supernatant was then disregarded, and the remaining volume of the cultures was added to each respective tube. The four tubes were once again centrifuged at 13krpm for 1 minute at 4°C. The supernatant was disregarded, and the pellet was vortexed until no clumps remained at the bottom of the tube. The vortexed pellets were then resuspended in 250µL of resuspension buffer and mixed by gently pipetting up and down, and by vortexing. Then 250µL of lysis solution was added to the microfuge tubes and mixed by inverting the tubes 4-6 times. The lysis solution was allowed to react for no more than 5 minutes with the mixture. After approximately 3-5 minutes, 350µL of neutralization solution was added to each tube, and the tubes were mixed thoroughly by inverting them ten times. The tubes were then centrifuged at 13krpm for 10 minutes at 4°C to pellet the chromosomal DNA. After centrifugation, the supernatant of each tube was transferred to a labeled prep spin column, without disturbing the pellet. The spin columns were centrifuged at 13krpm for 1 minute at 4°C. Flow through was discarded. 500µL of buffer PB was then added to the spin column and centrifuged at 13krpm for 1 minute at 4°C, the flow through was discarded. Then, 750µL of wash buffer was added to the column, and the tube was centrifuged at 13krpm for 1 minute at 4°C. After discarding of the supernatant, the empty column was centrifuged at 13krpm for 1 minute at 4°C. The supernatant was discarded, and the empty column was centrifuged again. After removal of the supernatant, the column was transferred to a sterile

labeled 1.5mL microfuge tube. 50µL of elution buffer was added to the column and allowed to incubate at room temperature for 2 minutes. Afterward, the tubes were centrifuged at 13krpm for 1 minute at 4°C, and the column was disregarded. Each sample was then measured by Nanodrop, using the elution buffer as a blank. 1µL of each sample, including the elution buffer, was used for Nanodrop. The data for ng/µL as well as A260/A280 and A260/A230 were recorded. The samples were stored in -20°C.

2.7 Sequencing the Isolated Plasmids

In order to confirm the identity of the isolated plasmids, the samples were sent for sequencing. To prepare samples, first, the primers were diluted to the needed concentration of 1.6 µM from their original stock concentration of 100mM. The primers were first diluted to a 10µM concentration, and then further diluted to the final concentration of 1.6 µM. For HXB-OJ41, two primers were utilized, a forward (HXD-C65) and reverse (HXD-C66). For HXB-OJ39, T7 primer was used. In total 3 tubes were prepared. Then, 4µL of the respective primer was added into each tube. Each primer is designed to be complementary to their respective plasmid (Table 1).

Table 1. Primer Details

Primer Name	Plasmid Designed For	Nucleotide Sequence	TM	GC content	Forward or Reverse
HXO-C65	pFastBac	GGATTATTCATACCGTCCCA	50	45%	Forward
HXO-C66	pFastBac	AATGTGGTATGGCTGAT	57	41%	Reverse
T7	pEt	TAATACGACTCACTATAGGG	57	40%	NA

Purified plasmids were added to the respective tubes along with autoclaved milipore water for a total volume of 12µL per tube (Table 2). The tubes were then sent to NCBI for sequencing and later compared to the known nucleotide sequence of both constructs.

Table 2. Reagents Added to Sequencing Tubes

Tube ZK774	**Component**	**Stock Concentration**	**Concentration Used**	**Volume Added**
	Autoclaved Millipore Water	NA	NA	3.1µL
	HXB-OJ39 Plasmid	162.9ng/µL	162.9ng/µL	4.9µL
	T7 Primer	100 µM	1.6 µM	4µL
Tube ZK775	**Component**	**Stock Concentration**	**Concentration Used**	**Volume Added**
	Autoclaved Millipore Water	NA	NA	6.8µL
	HXB-OJ41Plasmid	692.5ng/µL	692.5ng/µL	1.2µL
	HXO-C65 Primer	100 µM	1.6 µM	4µL
Tube ZK776	**Component**	**Stock Concentration**	**Concentration Used**	**Volume Added**
	Autoclaved Millipore Water	NA	NA	6.8µL
	HXB-OJ41Plasmid	692.5ng/µL	692.51ng/µL	1.2µL
	HXO-C66 Primer	100 µM	1.6 µM	4µL

2.8 Gel Electrophoresis

In order to further confirm the identity of the isolated plasmids, both samples were run in on a gel. The gel was prepared by first measuring out 50mL of 0.5X TBE buffer using a 100mL cylinder and transferring the liquid to a 500mL Erlenmeyer Flask. Then, 0.35g of agarose was weighed out and added to the flask. The mixture was heated in the microwave for two 45 second intervals and mixed in between until the solution was clear. The mixture was then poured into a

cast, and combs were added to create wells. The gel was allowed 20 minutes to solidify. The gel was then transferred to a gel electrophoresis tank. The tank was filled with 0.5x TBE buffer until it covered the gel. The first lane run was 10µL of Lambda phage DNA mixed with 2µL of GelRed dye. The second lane was 1.4µL of isolated plasmid HXB-OJ41 and 2µL of GelRed dye. The third lane was loaded with 6.2µL of isolated plasmid HXB-OJ39 and 2µL of GelRed dye. The gel was run for 75 minutes on 80V and analyzed.

Chapter 3. Results and Discussion

DH5 Alpha Cells were transformed with plasmids pFastBac GST C1C2BMunC2C, and pET28a C1C2BMunC2C to prepare the bacterial stocks HXB-OJ41 and HXB-OJ39 respectively (Figure 6). Plasmid pFastBac GST C1C2BMunC2C is known to carry antibiotic-resistant genes for Ampicillin, while plasmid pET28a C1C2BMunC2C is known to carry antibiotic-resistant genes for Kanamycin. The cells were then streaked onto LB media containing either Ampicillin (100µg/mL) or Kanamycin (50µg/mL) to serve as selective markers. After an incubation period of 24 hours, the strain HXB-OJ41 grown on Ampicillin was observed to have 21 individual colonies, while the strain HXB-OJ39 grown on Kanamycin was observed to over 100 individual colonies. Since DH5 Alpha cells do not naturally contain genes for antibiotic resistance to Ampicillin or Kanamycin, the presence of colonies on either plate indicate successful transformation with respective plasmids.

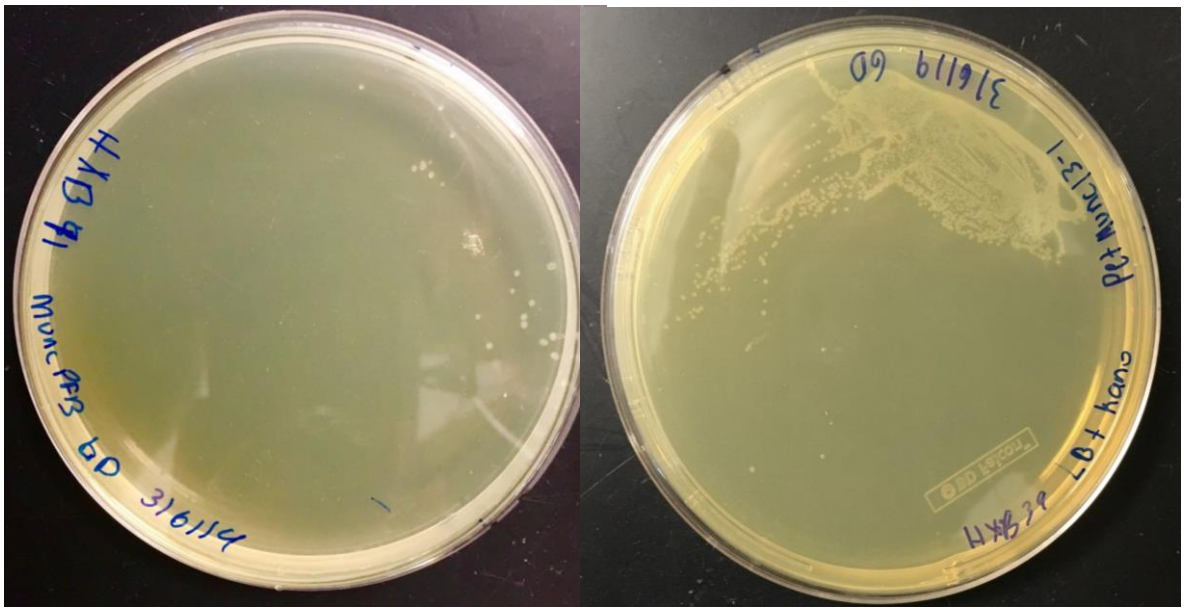


Figure 6. LB+Ampicillin (100µg/mL) plate with pFastBac/HXB-OJ41 colonies (left) and LB+Kanamycin (50µg/mL) plate individual pET/HXB-OJ39 colonies (right). 21 colonies were observed for HXB-OJ41 on Ampicillin, and over 100 colonies were observed for HXB-OJ39 on Kanamycin.

After the successful transformation of DH5 Alpha Cells with plasmids pFastBac GST C1C2BMunC2C and pET28a C1C2BMunC2C, plasmids were inoculated from the bacterial stock. The inoculated plasmids were placed in a shaking incubator overnight. From each of the two liquid inoculation cultures, the plasmid was isolated in two samples. From there, the elution buffer from the plasmid isolation kit was used as a blank, and the Nanodrop reading of each sample was recorded (Table 3).

When taking a Nanodrop reading, the ng/µL provides the actual concentration of the plasmid, while the 260/280 ratio and the 260/230 ratio are used to measure the purity of the sample. A sample is said to be pure with a 260/280 ratio of 1.8 and a 260/230 ratio of 2.0. For the HXB-OJ41 samples A and B, both exhibited high concentrations (Table 3). Likewise, both of their 260/280 ratios were very close to 1.8, as they were found to be 1.83(sample A) and 1.88 (sample B). Since these concentrations only had a difference of 1.6% and 4.4% from the ideal value of 1.8, it can be concluded that they are mostly pure. The A260/230 ratio for samples A and B of were also close to 2.0, respectively being 2.19 (percent difference of 9.5%) and 2.25 (percent difference of 12.5%) though slightly higher in differences (Table 3). However, since the excepted range of A260/230 is 2.0-2.2, these samples can still be considered pure. For HXB-OJ39, samples A and B had significantly lower concentrations of 160.4ng/µL and 168.3ng/µL respectively (Table 3). The A260/280 ratio of sample A and B was 1.9, with a 5% difference (Table 3). The A260/230 values for HXB-OJ39 samples A and B were also found to have higher contamination levels than HXB-OJ41, with a 12.5% difference and a14.1% difference from the ideal value (Table 3). This slight difference shows that there may be some minor contamination, but the products are still mostly pure.

Sample	ng/μL	A260/280	A260/230
HXB-OJ41 A	**692.9**	**1.83**	**2.19**
HXB-OJ41 B	**565.9**	**1.88**	**2.25**
HXB-OJ39 A	**160.4**	**1.9**	**2.25**
HXB-OJ39 B	**168.3**	**1.9**	**2.29**

Table 3. Concentrations, A260/280 ratios and A260/230 ratios of samples A and B from HXB-OJ41 and HXB-OJ39 plasmid isolates. The A260/280 and A260/230 values show the products are mostly pure, with possibly some slight contamination

Next, the samples were then run on gel electrophoresis on a 7% gel (Figure 7). The samples were all loaded with equal amount DNA, 1ng, and equal parts dye, GelRed, to ensure that all results were standard. A Lambda Phage Ladder was used, which is known to be 20k bp. The truncated Munc13-1 gene is known to be approximately 4.5kb in length. The plasmid pFastBac is known to have a length of approximately 4.5kb, while the plasmid pET is known to have a length of 5.2kb. Thus, this would have given both samples a total length of 9-10kb. When run on the gel using Lambda phage, the samples were compared to the gel. Sample HXB-OJ39 was noted to be only slightly above 9kb, with HXB-OJ41 being almost exactly at 9kb, which shows evidence for them both being the correctly transformed plasmids with the appropriate insert (Figure 7).

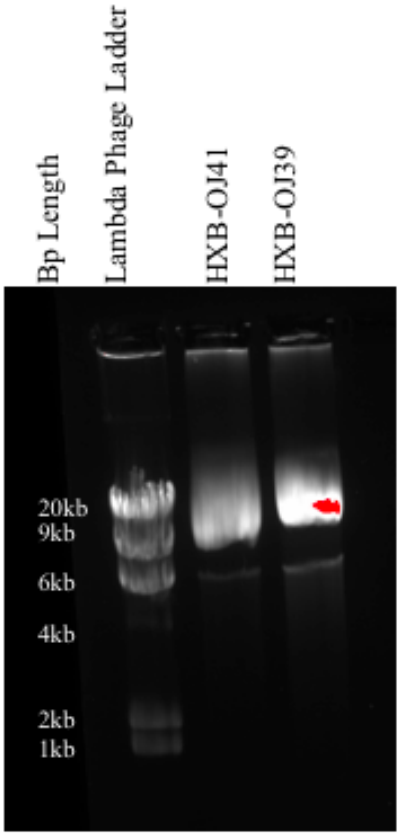


Figure 7. Gel Electrophoresis of HXB-OJ41 (sample A) and HXB-OJ39 (sample B) on 7% gel run alongside Lambda Phage DNA at 80V for 75 Minutes. The gel shows that HXB-OJ41 is present at 9kb, while HXB-OJ39 is present slightly above 9kb, at about 10kb.

After visual confirmation that the plasmids isolated from the bacterial cultures were the desired cultures, the two samples were sent for sequencing to further confirm their identities. The initial set of sequence primers used for HXB-OJ41 were designed to complement the pFastBac plasmid itself on either side of the multiple cloning sites. The first set of sequence data referred to the HXB-OJ41 plasmid with HXO-C65 primer (Figure 8). The data shows that of the 851 base pairs generated from sequencing, a strand of 154 base pairs showed similarity to the Munc13-1 construct from NCBI. Of that 154 base pairs sequence data (query) has a 94% similarity to the Munc13-1 construct with 146 base pairs being identical. 8 bases in the sequence are missing, or have not been sequenced, and 2 base pairs have been mutated to other bases, which 6 gaps in the total length. This high similarity confirms the identity of Munc13-1 in the plasmid.

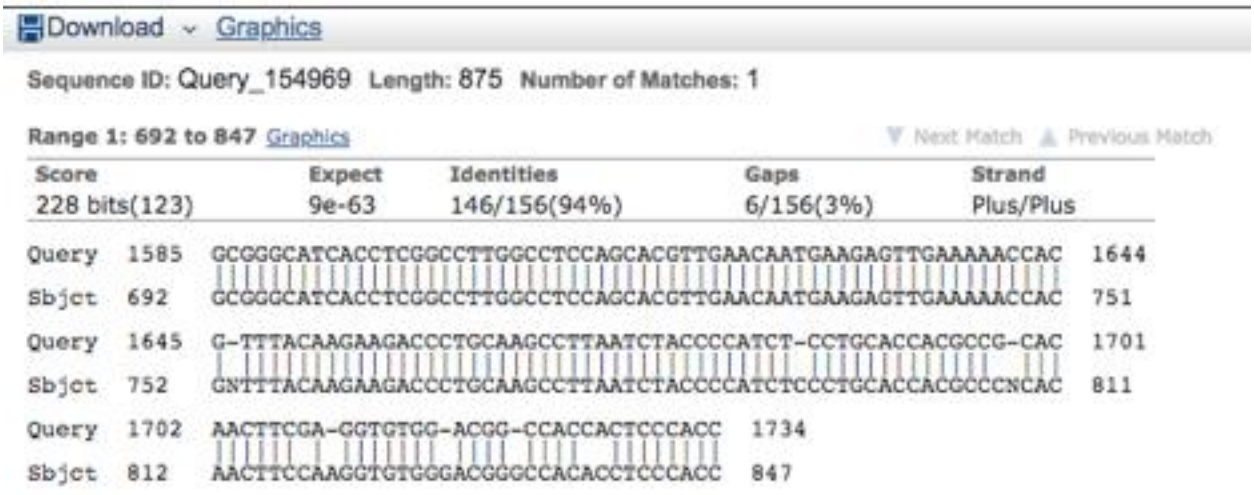


Figure 8. Sequencing results of HXB-OJ41 with HXO-C65 primer against Munc13-1 generated via Nucleotide BLAST on the NCBI website

For the sequencing results of HXB-OJ41 run with HXO-C65 primer, the data shows that 939 base pairs generated from sequencing showed almost identical similarity to the Munc13-1 construct from NCBI, in 3 different segments (Figure 9). The first segment had a strand of 556 base pairs, in which 555 were identical matches with one mutated base, for an overall similarity of 99%. The next two segments, one of which had a length of 241 base pairs and the other a length of 142, were both found to be 100% identical to the Munc13-1 construct on NCBI. No segments were found to have any gaps. This high similarity confirms the identity of Munc13-1 in the plasmid.

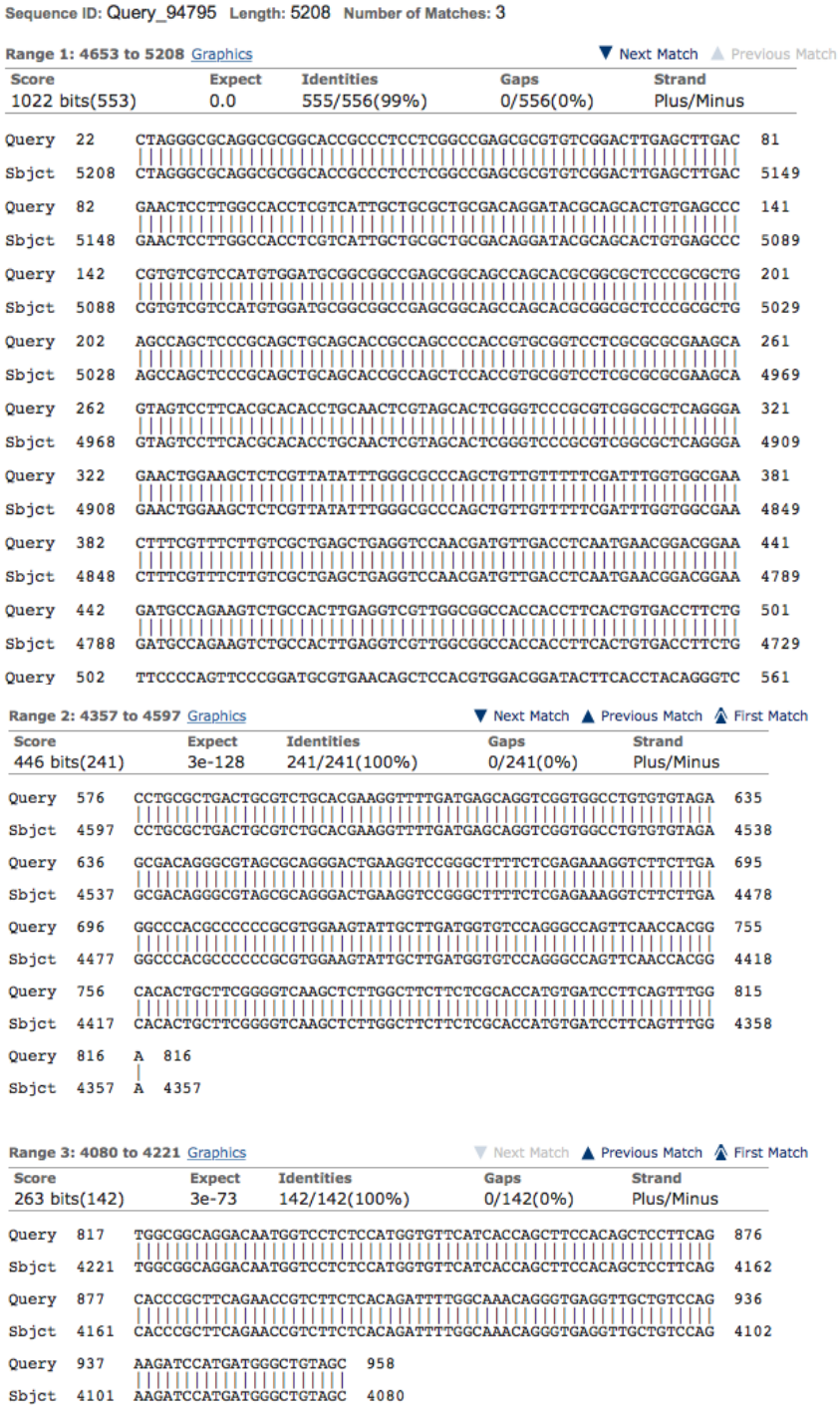
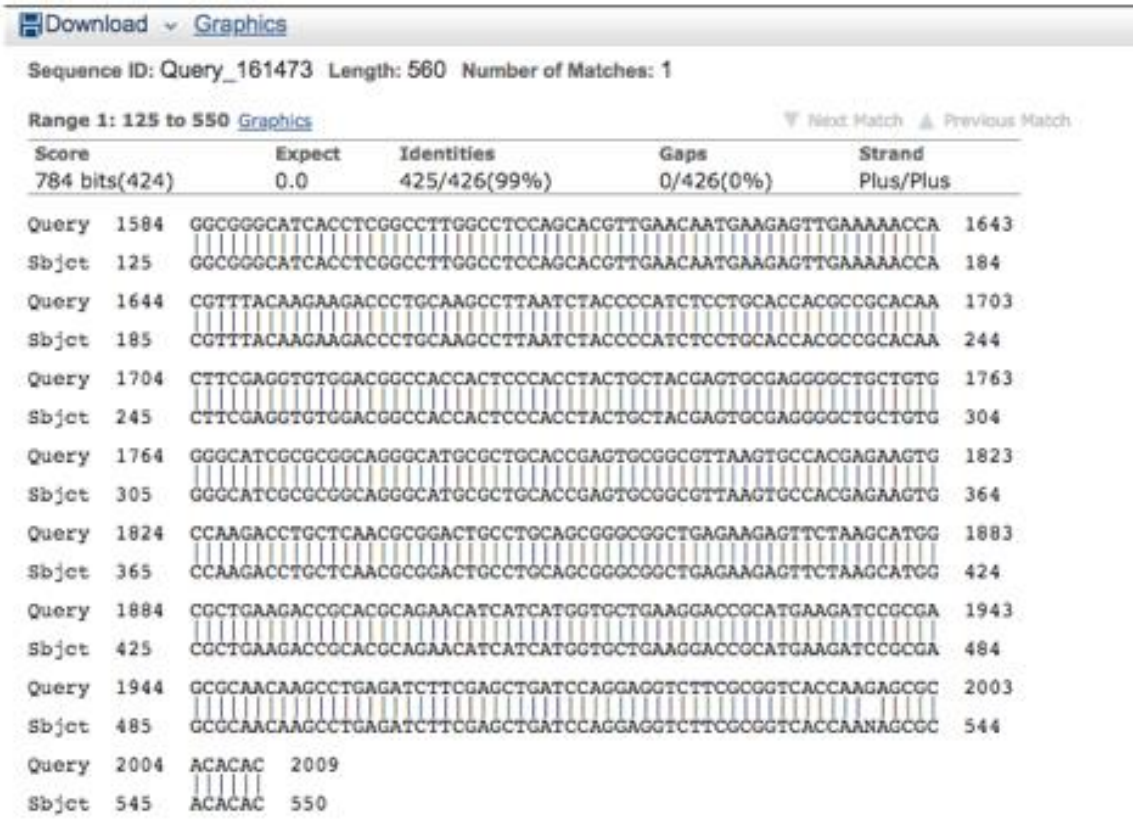


Figure 9. Sequencing results of HXB-OJ41 with HXO-C66 primer against Munc13-1 generated via Nucleotide BLAST on the NCBI website.

For the sequencing results of HXB-OJ39 run with T7 primer, the data shows that of the 550 base pairs generated from sequencing, a strand of 426 base pairs showed similarity to the Munc13-1 construct from NCBI (Figure 10). Of that 426 base pairs, sequence data (query) has a 99% similarity to the Munc13-1 construct with 425 base pairs being identical, with one base being mutated and no gaps present in the sequence. This high similarity confirms the identity of Munc13-1 in the plasmid.


Download Graphics

Sequence ID: Query_161473 Length: 560 Number of Matches: 1

Range 1: 125 to 550 Graphics

Score	Expect	Identities	Gaps	Strand
784 bits(424)	0.0	425/426(99%)	0/426(0%)	Plus/Plus

```
Query  1584  GGCGGGCATCACCTCGGCCTTGGCCTCCAGCACGTTGAACAATGAAGAGTTGAAAAACCA  1643
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125   GGCGGGCATCACCTCGGCCTTGGCCTCCAGCACGTTGAACAATGAAGAGTTGAAAAACCA  184

Query  1644  CGTTTACAAGAAGACCCTGCAAGCCTTAATCTACCCCATCTCCTGCACCACGCCGCACAA  1703
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185   CGTTTACAAGAAGACCCTGCAAGCCTTAATCTACCCCATCTCCTGCACCACGCCGCACAA  244

Query  1704  CTTCGAGGTGTGGACGGCCACCACTCCCACCTACTGCTACGAGTGCGAGGGGCTGCTGTG  1763
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245   CTTCGAGGTGTGGACGGCCACCACTCCCACCTACTGCTACGAGTGCGAGGGGCTGCTGTG  304

Query  1764  GGGCATCGCGCGGCAGGGCATGCGCTGCACCGAGTGCGGCGTTAAGTGCCACGAGAAGTG  1823
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305   GGGCATCGCGCGGCAGGGCATGCGCTGCACCGAGTGCGGCGTTAAGTGCCACGAGAAGTG  364

Query  1824  CCAAGACCTGCTCAACGCGGACTGCCTGCAGCGGGCGGCTGAGAAGAGTTCTAAGCATGG  1883
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365   CCAAGACCTGCTCAACGCGGACTGCCTGCAGCGGGCGGCTGAGAAGAGTTCTAAGCATGG  424

Query  1884  CGCTGAAGACCGCACGCAGAACATCATCATGGTGCTGAAGGACCGCATGAAGATCCGCGA  1943
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425   CGCTGAAGACCGCACGCAGAACATCATCATGGTGCTGAAGGACCGCATGAAGATCCGCGA  484

Query  1944  GCGCAACAAGCCTGAGATCTTCGAGCTGATCCAGGAGGTCTTCGCGGTCACCAAGAGCGC  2003
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485   GCGCAACAAGCCTGAGATCTTCGAGCTGATCCAGGAGGTCTTCGCGGTCACCAANAGCGC  544

Query  2004  ACACAC  2009
              ||||||
Sbjct  545   ACACAC  550
```

Figure 10. Sequencing results of HXB-OJ39 with T7 primer against Munc13-1 generated via Nucleotide BLAST on the NCBI website.

The slight differences in base pair sequence suggest that although it is likely that the sequenced plasmids are the correct constructs, it is expected that there are some mutations in the sequences or in the plasmids that occurred during transformation. Further work in the Xu lab to

continue this project will transform E.Coli or Sf9 with the purified plasmids, and additional sequencing may be done to confirm the presence of the truncated Munc13-1. After the plasmid is confirmed, protein isolation from the cell cultures will follow.

Munc13-1 has been determined to play an essential role in the regulation of membrane fusion and degranulation in both mast cells and neurons via its interactions with other proteins like Munc18 and the SNARE complex. A deeper understanding of the involvement of Munc13-1 in the SNARE complex assembly and disassembly is crucial to develop new treatments for the over or under regulation of membrane fusion and the potential threats and diseases it causes to the body.

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