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Localization of FAM171B mRNA Expression In Mouse Brain Using In-Situ Hybridization

Ashani Kumudika Sudasinghe
Minnesota State University, Mankato

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Localization of FAM171B mRNA Expression

In Mouse Brain Using *In-Situ* Hybridization

by

Ashani Kumudika Sudasinghe

A Thesis Submitted
In Partial Fulfillment of the Requirements
For the Degree of Master of Science in Biology
In Biological Sciences

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Localization of FAM171B in mRNA Expression in Mouse Brain Using *In-Situ* Hybridization

Ashani Sudasinghe

This thesis has been examined and approved by the following members of the student's committee.

Geoffrey Goellner, Ph.D.
Advisor

David Sharlin, Ph.D.
Committe member

Rachel Cohen, Ph.D.
Committee member

Abstract

Localization of FAM171B mRNA Expression

In Mouse Brain Using *In-Situ* Hybridization

Name - Ashani Sudasinghe

Degree- Masters of Science in Biology

University- Minnesota State University Mankato MN56001

Year- 2017

Proteins containing polyglutamine (polyQ) tracts within their primary amino acid sequence are particularly interesting because expansion mutation within them has been shown to underlie a growing list of severe neurodegenerative disorders including Huntington's Disease and several types of Spinocerebellar Ataxias. FAM171B is a novel polyQ protein that was originally identified via large scale sequencing efforts. However, to date, very little is known regarding its normal cellular function and expression pattern. In this study, *in situ* hybridization was utilized to assess whether FAM171B is expressed in developing (postnatal days 7, 21, 42) and adult mouse brains. The results of the experiments performed suggest that FAM171B is indeed expressed in the brain, with pronounced expression in the hippocampus, Purkinje cells of the cerebellum, and cerebral cortex. Given these findings, FAM171B should be considered a candidate gene for exploration of molecularly uncharacterized neurodegenerative diseases.

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Chapter 1

Introduction

While the human genome encodes approximately 20,000 proteins; a substantial number of them remain uncharacterized *(1)*. One such novel gene product is the 92kD protein FAM171B *(2)*. Although there is minimal published data regarding the molecular function of FAM171B in cells, some important characteristics can be inferred from the bio-informatic analysis of its amino acid sequence. For example, FAM171B is predicted to contain an A-T hook within its structure. Since A-T hooks are DNA binding domains, FAM171B may therefore have a nuclear function. Additionally, FAM171B contains a polyglutamine (polyQ) region within its primary amino acid sequence *(2)*. PolyQ proteins are particularly interesting, as mutations in these regions have been shown to underlie a growing list of severe neurodegenerative disorders *(3)*.

Polyglutamine disorders are classified by nine neurodegenerative diseases (Table 1). Severe neurodegenerative disorders such as Huntington's Disease, several Spinocerebellar Ataxias (SCA 1,2,3,6,7,17), Spinal-Bulbar Muscular Atrophy (SBMA), and Dentatorubral-Pallidoluysian Atrophy (DRPLA) are all caused by mutations in polyQ stretches within their respective gene products *(3, 4) (5)*. All polyQ diseases are progressive and ultimately fatal disorders without a known cure *(5)*. In each polyQ disorder, the glutamine sequence, which is coded for by cysteine, adenine and guanine as a trinucleotide (CAG) within the mRNA coding region, *(8)* extends well beyond its normal range leading to specific cell loss within the brain and consequent disease *(5) (6)*. An interesting feature regarding this phenomenon is that the longer the polyQ stretch within

the protein, the more severe the disease manifests -- clearly implicating the polyQ region itself as being the toxic agent *(5) (7)*. The damage results from the toxic gain of function of protein aggregates due to expansion of translated CAG repeats *(7) (9)*.

Table1. Polyglutamine disorders with normal and mutant CAG repeats & the brain regions that are affected *(10-19)*.

Disease	Type of nucleotide	Normal Range	Mutant range	Most affected Brain region
Huntington's Disease	CAG	11-34	37-121	Caudate, Cerebral Cortex
Spinocerebellar Ataxia type 1 (SCA1)	CAG	19-36	42-81	Cerebellar Purkinje cells brain stem
Spinocerebellar Ataxia type 2 (SCA2)	CAG	15-29	35-59	Cerebellar Purkinje cells brain stem
Machado-Joseph Ataxia (SCA3)	CAG	12-40	66-200	Cerebellar Dentate neurons, basal ganglia, brain stem
Spinocerebellar Ataxia Type 6 (SCA6)	CAG	4-16	21-28	Cerebellar purkinje cells, Dentate nucleus
Spinocerebellar Ataxia Type 7 (SCA7)	CAG	<64	>64	Brain stem. cerebellum
Spinocerebellar Ataxia Type 12 (SCA12)	CAG	9-28	55-78	Cerebellum
Spinocerebellar Ataxia Type 17 (SCA17)	CAG	29-42	47-55	Cerebellar Purkinje cells
Dentatorubral-Pallidoluysian Atropy (DRPLA)	CAG	7-34	49-83	Cerebellum, basal ganglia, Cerebral Cortex

Spinocerebellar ataxia type 1 (SCA1) (Table 1) is an inherited polyglutamine neurodegenerative disorder characterized by ataxia, the loss of voluntary controlled

coordination of muscle movements and progressive bulbar dysfunction *(10)*. Unaffected individuals possess ~6-39 CAG repeats within the SCA1 gene located on the short arm of chromosome 6 *(10)*. An affected individual, in contrast, can exhibit anywhere from 40 to 81 CAG repeats within the SCA1 gene *(10)*.

Spinocerebellar ataxia type 2 (SCA2) (Table 1) is an autosomal dominantly inherited neurodegenerative disorder that results from an expansion of CAG repeats within the ATXN2 gene *(11)*. SCA type 2 disorder, which causes damage primarily to cerebellar purkinje cells *(11)*, produces several symptoms, with progressive ataxia dominating. Unaffected individuals can exhibit from 14 up to 31 CAG repeats while affected persons are known to possess more than 32 CAG residues within the ATXN2 gene *(11)*.

Spinocerebellar Ataxia type 3 (Table 1), also known as Machado-Joseph disease, is the most commonly inherited Spinocerebellar ataxia. Persons diagnosed with this disorder exhibit 61-84 CAG repeats within their MJD1 gene, resulting in neuronal degeneration of the basal ganglia, brain stem, spinal cord and cerebellum *(12)*. Unaffected individuals, on the other hand, contain a much lower number (12-37) CAG repeats within the MJD1 gene *(12)*.

Spinocerebellar Ataxia type 6 (Table 1) primarily affects the cerebellum, in particular the purkinje cells *(13)*. Unaffected individuals exhibit 4-20 CAG repeats within the SCA6 gene while the diseased gene can possess from 21 up to 33 CAG repeats *(13)*.

Spinocerebellar Ataxia type 7 (Table 1) results in severe neuronal loss in the inferior olivary complex as well as damage to neuronal cells of the cerebral cortex *(14)*.

CAG repeats within the pathological gene can vary between 37 and 200 CAG repeats whereas in the normal SCA7 gene, there are less than 36 CAG repeats *(14)*.

Spinocerebellar ataxia type 12 (SCA12) is a slowly progressive neurodegenerative disorder that is dominantly inherited. Action tremors, anxiety and depression are the most common symptoms associated with this disorder *(15)*. SCA12 results from expansion of the CAG repeats within the PPP2R2B gene. Unaffected individuals contain 7-28 CAG repeats within this gene. Diseased individuals, on the other hand, can exhibit anywhere from 55-78 CAG repeats *(15)* (Table 1).

Spinocerebellar Ataxia type 17 (SCA17) is characterized by ataxia, dementia and involuntary muscle movements, resulting in chorea (uncontrollable motor dysfunction) and dystonia (involuntary muscle movements). Patients with this disorder start to express symptoms at mid age *(16)*. Neuronal damage occurs mainly within the cerebellum, brain stem and cerebrum. The TBP gene, which is affected by the number of CAG repeats present, encodes a TATA box binding protein that is necessary for transcription to take place *(16)*. Unaffected individuals contain 25-40 CAG repeats within the TBP gene whereas affected persons contain 41-48 CAG repeats within this gene *(16)* (Table 1).

Dentatorubral and pallidoluysian atrophy (DRPLA) is another polyglutamine neurodegenerative disorder that is characterized by ataxia and abnormal muscle control, but affected persons also experience tremors, seizures and even premature death. This disease is inherited in an autosomal dominant fashion and like SCA17 occurs in middle-aged individuals *(17)*. In these persons, neuronal damage and gliosis occur in many regions of the brain, although the Dentate nucleus of the cerebellum, the red nucleus, the Globus

pallidus and the sub-thalamic nucleus show prominent neuronal damage *(17)*. In unaffected individuals, the CAG repeat sequence within the atrophin-1 gene can vary between 6 to 35 CAG repeats (Table 1). Meanwhile, individuals with DRPLA can possess from 49 up to 88 CAG repeats within this gene *(17)*.

Huntington's disease is an autosomal dominantly inherited neurodegenerative disorder categorized by chorea, cognitive decline, dementia and ultimately death within 15-20 years of being diagnosed *(3)*. Interestingly, Huntington's disease is characterized by putative and progressive clinically irreversible symptoms involved with neural protein aggregates *(18)* that are caused by polyglutamine expansion within the Huntingtin (HTT) gene on the short arm of chromosome 4. Unaffected individuals typically contain 26 or fewer polyglutamine repeats within the HTT gene while affected persons exhibit between 40 and ~100 CAG repeat sequences (Table 1). In the later group, the disease usually manifests itself in mid-life but the symptoms may not appear until much later in life due to reduced penetrance of the CAG repeat regions. It is interesting to note that as the Huntingtin gene is passed on to the next generation from an affected parent (50% chance of transmitting the abnormal HTT gene to his/her children *(19)*), the polyglutamine regions can expand. The severity and manifestation of the disease, however, is directly dependent on the number of CAG repeats in the HTT gene: (1) 27-35 CAG repeats – these individuals do not exhibit Huntington's disease but there is a high probability of them transmitting the disease to the next generation, (2) 36-39 CAG repeats – these persons may or may not be affected by Huntington's disease due to reduced penetrance, and (3) ≥40 CAG repeats – these individuals will always exhibit Huntington's disease *(19)* (Table 1).

According to Nelson *et. al. (21)*, expansion of polyQ stretches somehow endows proteins with a toxic gain of function that ultimately kills neurons. Even though each polyglutamine disease is rare, as a group these are the most common form of inherited neurodegenerative diseases *(7)*.

Mesenchymal stem cells, also known as marrow stromal cells (MSC), have been used as a therapeutic tool for cell therapy as well as in many clinical trials that have been approved by the FDA *(22)*. Myocardial infarction, strokes and autoimmune disorders are a few of the many disorders that MSCs have been used as a tool for therapeutic advantage. Nowadays, researchers are showing a great interest in determining whether or not MSCs can be used to treat neurodegenerative disorders, especially diseases like Huntington's disease that are progressive and fatal *(22)*. Pre-clinical trials suggest that transplantation (via intracerebral or intrathecal injection) of mesenchymal stem cells into the brain endorse endogenous neuronal growth, decrease apoptosis, and increase paracrine secretions that can be used to regenerate synaptic connections of already damaged neurons and control inflammation *(22)*. As such, mesenchymal stem cells can provide a potential regenerative approach for those progressive and fatal neurodegenerative disorders. Ongoing clinical trials where MSC's were directly injected in to the central nervous system during traumatic brain injuries support the current hypothesis regarding the use of MSCs as a therapeutic tool for neuronal cell regeneration in the brain *(22)*.

Since FAM171B also includes a PolyQ stretch within its protein, it is interesting to further investigate about this uncharacterized protein. Although it is not clear whether the newly identified polyQ protein FAM171B will ever be linked to a particular disease, polyQ

proteins in general are fascinating because of their common role in neurodegenerative disease. Moreover, uncovering the normal cellular function of a novel human protein, in this case FAM171B, is a worthwhile endeavor that will advance the field of cellular biology. Thus, the current project was undertaken to gain insight about the novel polyQ protein FAM171B, and specifically answer questions regarding which body tissues it is normally expressed in. If our findings show that FAM171B is expressed in the brain, then FAM171B could potentially be considered a candidate gene for exploring a molecularly uncharacterized neurodegenerative disease.

The methodology that was employed to accomplish the abovementioned goal, is a method coined *in-situ* hybridization. This specialized staining technique relies on Watson and Crick base pairing between polynucleotides within its complementary RNA sequence *(23)*. *In-situ* hybridization is a standard molecular technique that harnesses the power of nucleotide base pairing that occurs between complementary bases in RNA, to investigate for expression of specific genes in tissues of the body. Additionally, this technique can be highly sensitive when a targeted nucleic acid sequence is in a small percentage of cells or in specific regions of an organ *(24)*. *In-situ* hybridization is commonly used in cell biology to localize mRNA via light microscopy *(23)*. Hybridization products are obtained via a phosphatase coupled antibody reaction against the degoxygenin products *(25)*. Following sample fixation and embedding procedures, the sample can be analyzed using a light microscope to visualize gene expression *(24)*. In-order-to observe and analyze the expression of FAM171B mRNA in brain tissue, brains from mice representing four different age groups were harvested. Mice brains were used in this study given that

genomic sequencing data suggests that mice contain a FAM171B protein that is ~ 88% identical in sequence to the human FAM171B protein although the number of polyglutamine repeats does vary (16 in humans versus whereas 7 in mice) *(39)*.

Hypothesis

In the current study, it is hypothesized that FAM171B will be expressed in the brains of mice and that the expression may be temporally or spatially specific. To test this hypothesis, *in-situ* hybridization will be performed on various regions of brain tissue harvested from laboratory mice representing four different age groups.

Prediction

Observation of FAM171B is anticipated in the different regions of the mouse brain, although the expression of this protein may vary between the different regions of the brain. Expression of FAM171B is also expected to vary in a time-wise manner of the mouse life cycle, thus we will be able to detect this time-based expression by examining the brain tissue of mice belonging to different age groups.

Chapter 2

Methods and Materials

2.1 FAM171B Amplification

PCR was conducted to amplify FAM171B cDNA from mouse cochlea to insert the gene product in to a pGEM-T Easy vector. 12.5µl of Promega gotaq master mix (2X), 1µl of appropriate primers (10mM) (Primer sequences shown in Figure 4, Appendix), 2µl of DNA template (Cochlea cDNA) and 8.5µl H_2O were mixed. Reaction PCR cycle ran in 95°C for 2 min, 95°C for 30sec, 55°C for 30 sec. Reaction was extended at 72°C for 1 min. This reaction was repeated for 30 cycles. Reaction was then kept in 72°C for 5 min and stored at 4°C.

PCR products were analyzed by agarose gel electrophoresis (Figure 1, Results) and sent for sequence verification (Appendix, Figure 5 and 6). PCR clean-up was performed accordingly to manufacturer instructions (Quiagen). In short, pH should be less than 7.5 for PCR purification. Buffer PB, DNA and pH indicator was added to the spin column. Supernatant was discarded after centrifuging at 16000xg for 1 min. Buffer PE (750µl) was added to the spin column and centrifuged for 1 min. The microcentrifuge tube was replaced after another 1min centrifuge and supernatant was discarded. 30µl of the eluting buffer was added to the spin column with DNA and incubate 1min. Flow through was collected after 1 min centrifugation.

2.2 Gel Confirmation of FAM171B Insert Generation

1% Agarose gel was made using 0.6g of Agarose in 60ml of 1% TAE buffer and by adding 1.2µl Ethidium Bromide. 2µl of 6X-loading dye was added to the prepared 10µl DNA samples. 12µl of 1Kb bio-life DNA ladder (NEB) was inserted to the 1st well of the gel. Gel electrophoresis was conducted in 96V for 45 Min (Figure 1, Results).

2.3 Sub-clone FAM171B into a pGEM-T Easy Vector

TA cloning was performed using 7.5µl 2X rapid ligase buffer, 1 µg pGEM-T Easy vector, 0.5µl PCR product (DNA insert), 1.5µl T4 DNA ligase was mixed in micro centrifuge tubes and stored at 4°C overnight. (Appendix figure, 1)

2.4 Transformation

LB/Ampicillin/ IPTG/X-Gal plates were prepared. Ligation reaction mixture was centrifuged and 1.5µl of each ligation reaction was added to a sterile 1.5ml tube. JM109-L2005 high efficiency competent cells were placed in an ice bath until just thawed (5min). Cells were mixed by gently flicking the tube and 25µl of cells were transferred to the ligation reaction tubes. Mixture was gently mixed and incubated on ice for 30mins. Cells were heat-shocked for 25 Sec in a water bath at 42°C. Tubes were then immediately returned to the ice for 2 min. 450µl of room temperature LB medium was added to the ligation reaction transformation and incubated for 60mins at 37°C. 100µl of each transformation culture was then added to LB/Ampicillin/IPTG/X-Gal plates. Plates were incubated overnight at 37°C, and only the white colonies were selected (Figure 2, Appendix).

5ml of LB broth and 5µl of ampicillin were mixed to make liquid bacterial cultures. One isolated white colony was inserted to the mixture and incubated at 37°C for 18 hrs. For glycerol stocks, 1ml of culture was added to the 1.5 ml storage tubes and bacteria were pelleted. 25% glycerol and fresh LB broth was added to the tube, and glycerol stocks were stored at -80°C.

2.5 Isolating the vector with FAM171B gene

A quiagen mini-prep was used to isolate pGEM T-easy vector containing FAM171B according to manufacturer instructions. In short, 1 ml of overnight cultures were centrifuged and LB broth was decanted. Pelleted cells were re-suspended in 250µl buffer P1, and transferred to a new tube. 250µl buffer P2 was added and the tube was mixed thoroughly by inverting 4-6 times. 350µl buffer N3 was added and mixed immediately by inverting the tube 4-6 times. The mixture was centrifuged for 10min at 16,000xg. Supernatant was applied to the QIAprep spin column and centrifuged for 60sec, and the flow through was discarded. 0.5ml of wash buffer PB was added to filter, centrifuged for 60sec, and the flow through was discarded. 0.75 ml buffer PE was added as a wash buffer, centrifuged for 60sec, and the flow through was discarded. The mixture was centrifuge for additional 1min to remove residual wash buffer. To elute the DNA, the QIA prep column was placed in a clean 1.5 ml micro centrifuge tube, 50µl buffer EB was added to the center of each QIAprep spin column and centrifuged for 1 min.

2.6 Confirmation of Probe

Restriction Digest using NotI

NotI restriction enzyme (NEB) was used to cut the plasmid from SP6 and T7 promoter regions and to isolate the gene product (800bp). This step was performed to confirm that the plasmid contains FAM171B gene. Restriction digest was performed by adding 5µl of 10X BSA buffer, 300µg mini-prep DNA template, 5µl 10X NEB 3.1 buffer, 5µl NotI, 25µl of H_2O, and the reaction digested overnight at 37°C. Appendix figure 3 shows the confirmation of FAM171B after Restriction digest with NotI.

After restriction digest using NotI, DNA samples were sent for sequencing (Genewiz) and sequencing data confirmed FAM171B DNA insert (Appendix figure 5 and 6).

2.7 Linearization of FAM171B vector for Probe Generation

Restriction Digest ApaI (50µl) reaction

Restriction Digest at Sp6 promoter region of pGEM T-Easy vector was conducted using ApaI restriction enzyme. 5.6µg of DNA template, 2µl of ApaI (NEB), 5µl of Cut smart buffer (10x) and H_2O (up to 50µl) were mixed in a 1.5 micro-centrifuge tube and incubated at 25°C for overnight.

Restriction Digest using SalI 50µl reaction

Restriction Digest at T7 promoter region of pGEM T-Easy vector was conducted using SalI restriction enzyme. 5.6µg of DNA template, 2µl of Sal1 (NEB), 5µl of Buffer 3.1(10X) and H_2O (up to 50µl) were mixed in a 1.5ml tube. The reaction mixture was digested overnight at 37°C.

Agarose gel electrophoresis was performed on a small portion (4µl) of restriction reactions to confirm plasmids were fully cut with SalI and ApaI restriction enzymes (Appendix Figure 7). The rest of the restriction reaction (46µl) was then purified using a QIAquick cleanup kit. In short, 230µl of PB was added to 46µl of restriction mix. The reaction mixture was then placed to the QIA column and centrifuged for 60sec. After discarding the flow through, 750µl of buffer PE was added to the QIA quick column, centrifuged for 1 min, the flow through was discarded, and the column centrifuged for another 1min. To elute DNA, the QIAquick column was placed in a clean 2ml collection tube, 30µl buffer EB was added, and allowed to stand for 1min and eluate was collected by centrifugation. FAM171B purified digests were stored at -20ºC.

2.8 *In-vitro* RNA Transcription Reaction

RNA transcription was performed by adding DNA template (2µg), 5x transcription buffer (5.0 µl), 10mM DIG NTP mix (2.5 µl), RNAse inhibitor (1.0µl) and RNA polymerase SP6 or T7 (5.0 µl); water was added to adjust the final volume to 50µl. The solution containing DNA was then incubated at 37ºC for 2 hours. 2 µl of DNaseI (RNAse free) was added to the solution and incubated for 1 hour at 37ºC. A portion of the samples were then electrophoresed on 1% agarose gel in 1xTAE to confirm the presence of RNA probe, and that the DNA plasmid had been completely digested (Appendix Figure 8).

SP6 and T7 promotor regions of the pGEM-T Easy vector and ApaI and SalI restriction enzymes were used to generate FAM171B experimental (Antisense) and FAM171B control (Sense) probes. Orientation of FAM171B was determined by DNA sequencing data (Appendix Figure 5 and 6). Thus, mRNA transcribed after vector cut at

SP6 promotor region was chosen as the antisense (Experimental) probe and mRNA transcribed after vector cut at T7 promotor region was chosen as the sense (Control) probe (Appendix Figure 6).

Fragmenting RNA Probe:

A heat block was set at 60ºC. 50 µl of 0.2M Na_2CO_3 and 50 µl of 0.2M $NaHCO_3$ (final pH=10.2) was mixed to make hydrolysis buffer. 100µl of the probe was then added to 100µl hydrolysis buffer and the mixture was incubated at 60ºC for 10min and placed on ice.

To precipitate the RNA, 30 µl 2M Na acetate (pH=6.0) and 2.5 vol of 100% ethanol was added to each sample with each probe and incubated at -80ºC for an hour. The reaction was vortexed and centrifuged at 16000xg for 30 min at 4ºC and ethanol was decanted. The precipitated RNA was then washed with 1 ml of 70% ethanol. Reaction was then centrifuged at 16000xg for 10 min at 4ºC and the pellet dried. The pellet was then re-suspended in 50µl high molecular grade H_2O. The concentration was measured using the Nano-drop and the probe re-suspended at 20ng/µl in Hybridization buffer.

2.9 Mice Brains

C57BL/6 mice colonies were reared (IRB: 15-02), and brain tissues were dissected from male mice of 4 different age groups: p7, p21, p42, and 6 months. Brains were snap frozen in liquid nitrogen and stored at -80°C until further processing. Frozen brains were sliced using a cryostat, and 12um sections were deposited onto charged glass slides (Fisher labs). Adult mouse brains were approximately 8mm wide. Since this study focused on the

left side of the brain, and sagittal brain sections were collected every 100 microns, each adult mouse brain produced ~40 sections.

These thin sections of mouse tissue (that theoretically contain FAM171B) were then probed for indications of FAM171B mRNA expression.

2.10 *In-situ* Hybridization for Mice Brain Tissues

Day 1

Pre-hybridization.

Slides containing brain sections were fixed in 4% PFA / 1x DEPC-Tx PBS for 10min, and washed twice in 1x DEPC-Tx PBS for 2 min.

Tissue slices were acetylated in 0.1M TEA/0.25% Acetic Anhydride 10min. Next, tissues were permeablilized in 0.1% Triton X-100/PBS at RT for 10 min, and washed 3X with 1x DEPC-Tx PBS for 2 min using coplin jars. Slides were transferred to a hybridization chamber and 500 µl of hybridization buffer (20ml of formamide, 8ml of 20X SSC, 0.8ml 50X Denhardts reagent, 8ml of 50% Dextran sulfate, 1.2ml DEPC water, 2ml of fish sperm DNA) was added to the slides and incubated at room temp 2 hr. Probe dilutions were made in hybridization buffer. To make 500ng/mL of probe, 125µl of 20ng/µl stock probe was mixed with 5ml of Hybridization buffer. Probe was then heated at 80ºC 5min to denature. Hybridization buffer already on the slides was removed and 250µl diluted probe was then added to slides, and cover-slipped. Slides were hybridized overnight at 57ºC after slides were placed in a tray containing paper towels moistened with 50% formamide/0.2X SSC.

Day 2

Post hybridization

Coverslips were removed by allowing them to slide off while in 1x SSC buffer. 0.2xSSC was used to wash slides 4 times with 15mins intervals for 1 hr. at 68-72ºC in Coplin jars. Slides were then allowed to cool to room temperature, and washed with fresh RT 0.2xSSC for 5min.

Antibody and alkaline phosphatase detection with BCIP/NBT

Slides were washed in maleic acid buffer (MB) for 5min at room temperature. MB was removed and 30ml of 2% MBMB was added at RT for 1 hr. This blocking buffer was prepared by diluting 10% MBMB (Roche) 1x maleic acid buffer. Slides were removed individually and placed on the humidified chamber and covered with 500µl anti-Digoxygenin-AP antibody (Roche) diluted 1:2000 in 2% MBMB. Slides were incubated at room temperature for overnight.

Day 3

Post-antibody wash

Slides were washed in MB for 30 min at room temp in Coplin jar. Then slides washed in color reaction buffer (100ml 0.5M Tris pH 9.5, 10ml 5M NaCl, 2.5ml of 1M $MgCl_2$, 50µl Tween 20, and 387ml H_2O) for 30min. NBT/BCIP color reaction solution was made by mixing 200ul NBT/BCIP stock and 10 ml of color reaction buffer. 500 µl of NBT/BCIP color reaction mixture was added to each slide. Slides were incubated on a tray with dampened paper towels, covered in aluminum foil, and incubated 7 hours.

Day 4

Mount slides

Slides were washed 1xPBS for 5min at room temperature, and mounted using Aqua/Polymount (Polysciences). Once dry, slides were stored in at room temperature.

2.11 Analyzing microscopic slides and densitometry

After each *in-situ,* microscopic slides with mice brain sections were analyzed using a ZEISS Axiostar plus compound light microscope. Pictures of the experimental brain regions and control brain sections were taken using a progress C10 plus camera connected to the Axiostar plus compound light microscope.

Densitometry was used to conduct the semi-quantitative analysis of the expression of FAM171B in different regions of the mouse brain. Two main brain regions (hippocampus and cerebellum) were selected based on the highest expression of FAM171B. ImageJ-NIH software was used to convert each image to 8-bit to get a grey scale picture. In each microscopic picture, the chosen brain region with the FAM171B expression was highlighted and selected using the "analyze" tool. Expression of FAM171B in each experimental brain region was measured and background stain (from nearby "white/non-stained" region in same image) was subtracted from original experimental measurement. Control brain images (stained with "control" probe) from corresponding regions were obtained and measured in an analogous fashion. The control brain measurements were then subtracted from the measurement obtained from the experimental brain region, and the results plotted in bar graph format.

2.12 Statistics

Three mouse brains were collected for each age group (N=3), and each brain was micro-sectioned into 12 um sagittal brain sections. From each brain, ~40 brain sections were obtained for the experimental probe, and 10 brain sections were obtained for the control probe. Thus, after three *in-situ* experiments, 120 brain sections per age group were analyzed both semi-quantitatively and qualitatively and compared to 30 control brain sections per each age group. Specifically, this analysis was performed by measuring FAM171B expression (in Ammon's horn, Dentate gyrus, and cerebellum) in all experimental brain sections via densitometry (described above) ensuring that, the background staining of this region of the control samples was subtracted from the expression observed in experimental brain sections. Once the background was removed, and the expression level determined, the relative FAM171B expression of other age groups was normalized to P7. A univariate ANOVA test was then performed to determine whether or not the level of FAM171B expression was significantly different amongst the 4 age groups.

2.13 Qualitative study

A qualitative study was performed by observing the expression of FAM171B-mRNA throughout the brain, specifically focusing on the Hippocampus, Cerebellum, Cortex, Amygdala, Thalamus, Caudate and Corpus callosum. For this part of the study, a compound microscope was utilized to examine the respective brain regions, and a qualitative score was ascribed for each region by visually comparing the experimental signal versus the control signal. Expression levels were assigned as follows: high signal

(+++), medium signal (++), low signal (+), and no signal (-). For this part of the study, 120 experimental brain sections per age group were compared against 30 brain sections per age group.

Chapter 3

Results

3.1 Confirmation of FAM171B in Mouse Cochlea

FAM171B DNA was sucessfully amplified using mouse cochlear DNA as the cDNA template. Electrophoresis and subsequent comparison to a 1 kb DNA ladder (Lane 1) illustrate the presence of the desired 800bp fragment (Lanes 2 and 3). DNA sequence confirmed the PCR fragment was indeed FAM171B (Figure 6, Appendix). These results strongly suggest that FAM171B is indeed expressed, at the mRNA level, in mouse.

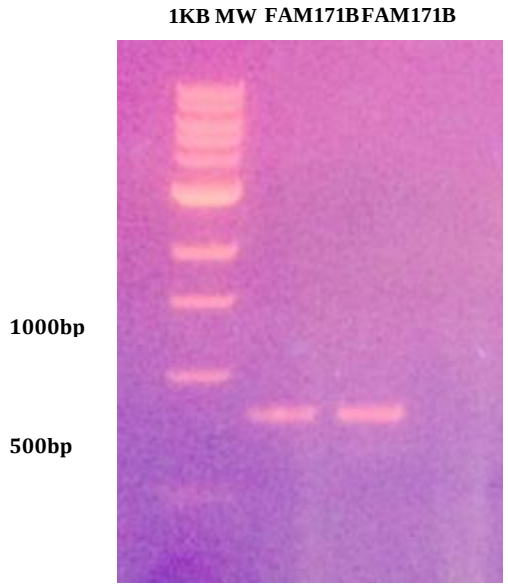

Figure 1. Gel electrophoresis analysis confirming the presence of FAM171B in mouse cochlea. 1kb DNA ladder on the first lane. FAM171B DNA bands on the second and third lane.

3.2 FAM171B mRNA expression in mouse brain

In-situ hybridization strongly suggests that, FAM171B mRNA is expressed widely in mouse brain (Figure 2). In this figure, the presence of the purple color throughout the brain tissue confirms the expression of FAM171B at the mRNA level after performing an *in-situ* hybridization using the experimental probe. Even though, FAM171B is expressed throughout the majority of the brain, expression is more prominent (denoted by the darker purple color) in specific regions of the brain including the hippocampus, cortex, cerebellum, thalamus, caudate and amygdala (Figure 2).

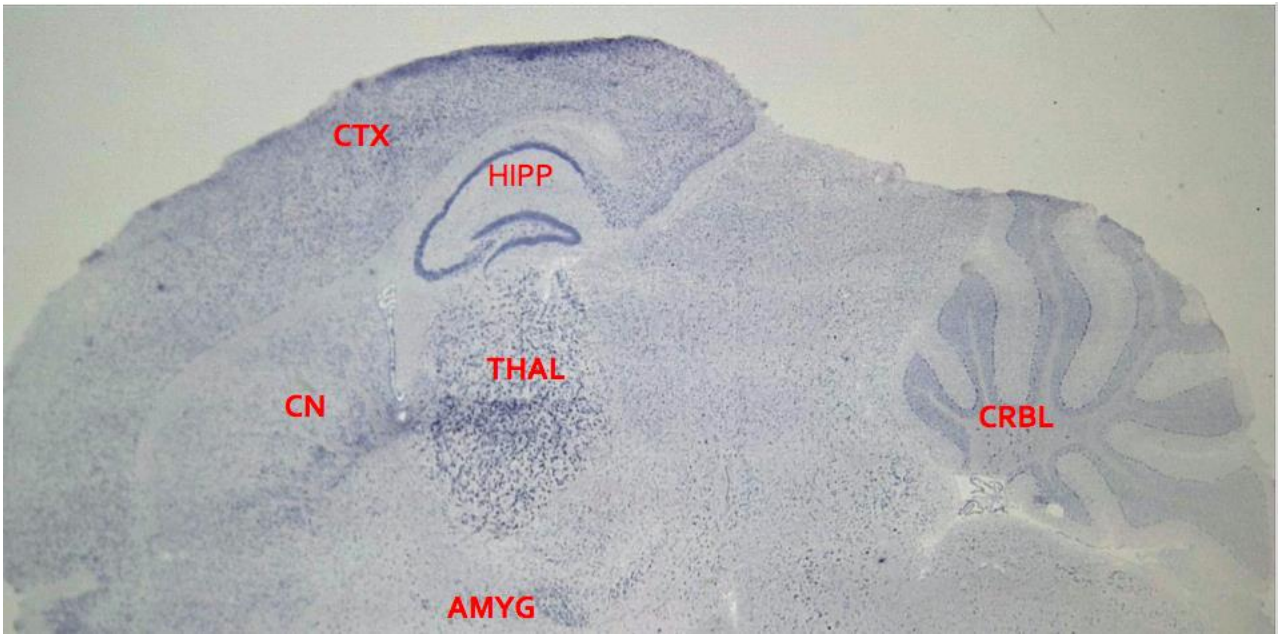

Figure 2. Sagittal brain section of a 21-day-old male mouse after performing *in-situ* hybridization with the experimental FAM171B mRNA probe. The image was obtained using a dissecting microscope on 10X magnification. HIPP correspond to the hippocampus, CTX stands for cortex, CRBL stands for cerebellum, THAL stands for thalamus, CN stands for caudate, and AMYG stands for amygdala.

3.3 Qualitative Analysis of FAM171B mRNA in mouse brain

Qualitative analysis of the different regions of the mice brains confirmed a high level of FAM171B mRNA expression in all four age groups examined (Table 1). As anticipated, FAM171B was expressed throughout the mice brains of the experimental group as compared to those sections hybridized with the control probe, which revealed no expression. Although FAM171B mRNA expression was widespread in the brains of all 4 age groups, much higher levels of expression were observed in the following regions: Dentate gyrus and Ammon's horn of the hippocampus, Purkinje cell layer and granular layer of the cerebellum, motor and sensory cortices, amygdala, thalamus and the caudate. Interestingly, expression of FAM171B was absent in the the white matter of the brain, such as Corpus callosum.

Table 1. Qualitative analysis of the expression of FAM171B mRNA in the different regions of the brains of mice belonging to different age groups after performing an *in-situ* hybridization. The +++ indicates the highest degree of expression, ++, moderate expression, +, low expression, and –, no expression.

Brain Structures	P7 Experimental	P21 Experimental	P42 Experimental	6M Experimental
Ammons Horn	+++	+++	+++	++
Dentate Gyrus	+++	+++	+++	++
Caudate	++	++	+	++
Cerebellum	++	++	+	+
Thalamus	++	++	+	++
Amygdala	++	++	+	++
Corpus Callosum	-	-	-	-
Motor Cortex	++	+++	++	++
Sensory Cortex	++	+++	++	++

3.4 Detection of FAM171B mRNA Expression in Hippocampus

Figure 3, illustrates FAM171B expression in the hippocampus of mice brains hybridized with the experimental (top panel) and control (bottom panel) probe. These data clearly indicate that FAM171B mRNA is strongly expressed in the hippocampus. All of the mice brains within the experimental group, regardless of age, exhibit a dark purple color following performance of the *in-situ* hybridization which suggests that FAM171B mRNA is highly expressed in this region. Control mice brains, on the other hand, (treated with a control FAM171B probe that contains the same exact mRNA sequence as the mRNA present in mice brain tissues), displays little to no purple color after performing the *in-situ* hybridization (Figure 3).

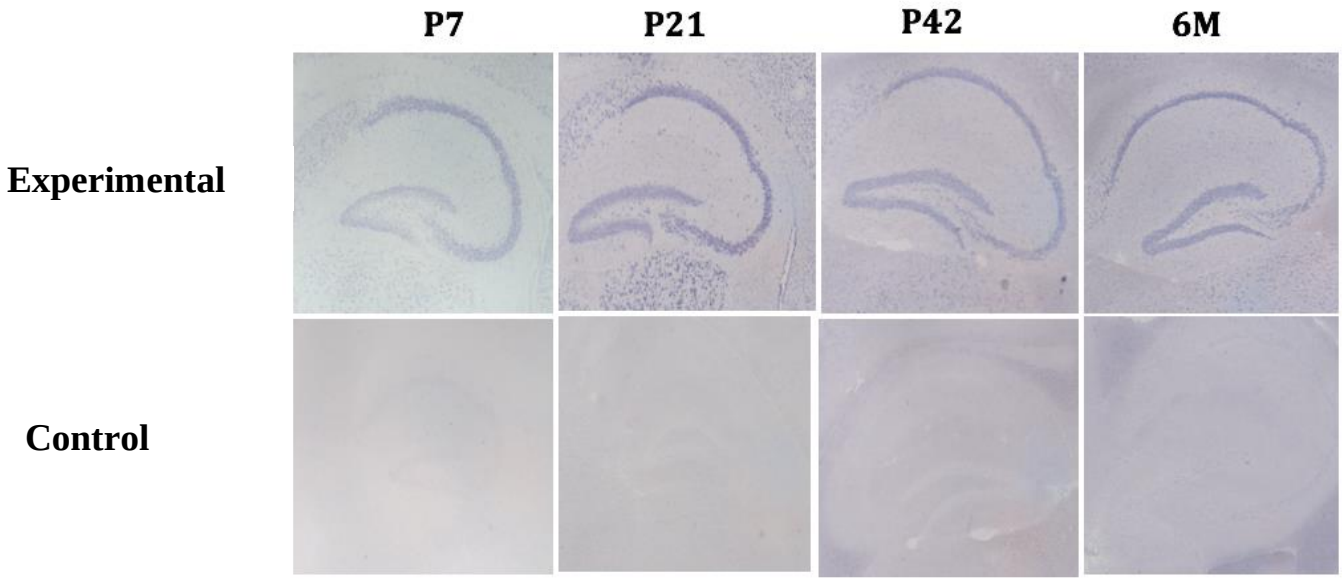

Figure 3. FAM171B expression in the hippocampus of 7-day, 21-day, 42-day, and 6-month-old mice. The image was observed using the 5X objective of a compound microscope.

Figure 4 illustrates prominent expression of FAM171B mRNA in the hippocampus of a sagittal brain section of a 21-day-old mouse model. This figure was captured after performing an *in-situ* hybridization of mice tissue using the experimental probe. The dark purple color indicates that FAM171B is highly expressed in both the Dentate Gyrus and the Ammon's Horn regions of the hippocampus.

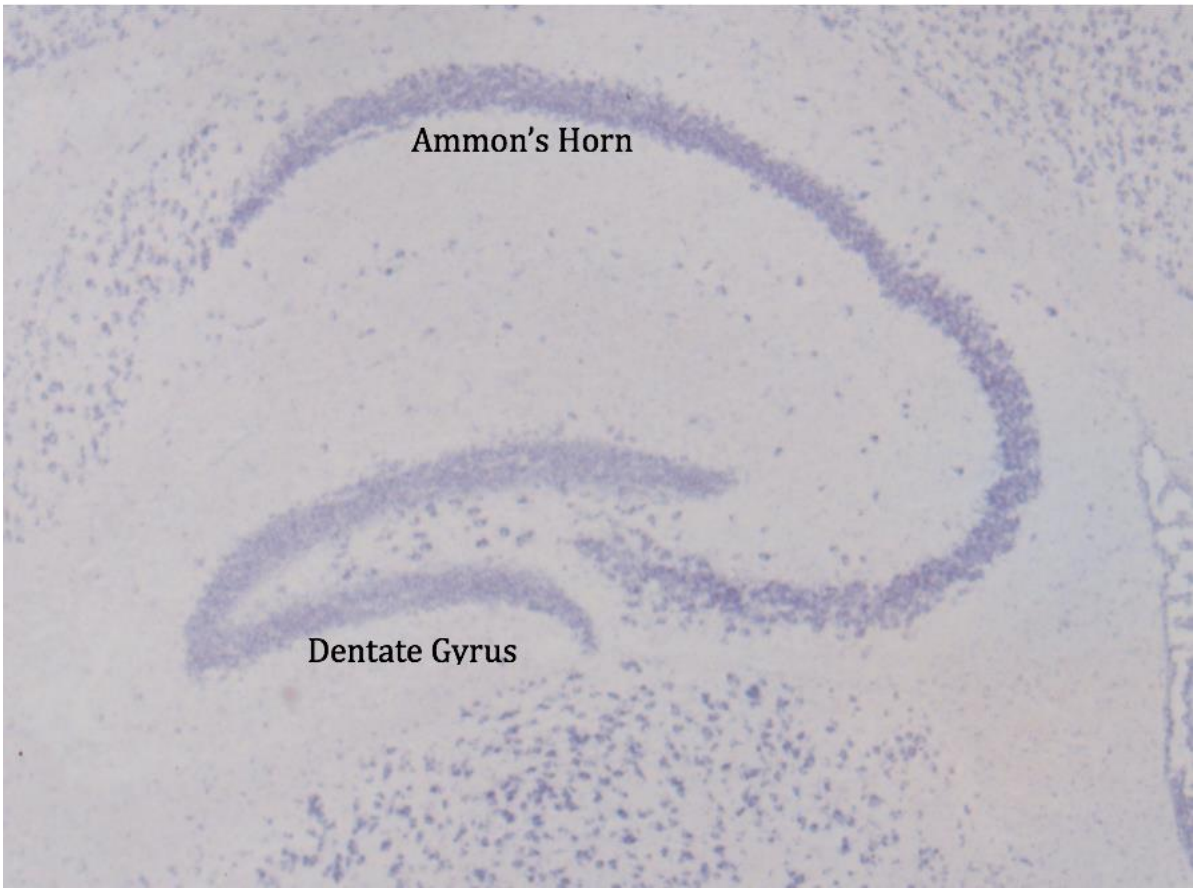

Figure 4. FAM171B expression in the Dentate Gyrus and the Ammon's Horn regions of the hippocampus of a 21-day-old mouse sagittal brain section hybridized with the

experimental probe. This image was observed using the 5X objective lens of a compound microscope.

Semi-quantitative analysis also indicates that FAM171B mRNA is highly expressed in the Ammon's Horn region of hippocampus of 7-day, 21-day, 42-day, and 6-month old mice brains hybridized with the experimental probe (Figure 5). A univariate ANOVA test displayed a P value (P = 0.573) suggesting that the expression of FAM171B is not significantly different amongst the four age groups.

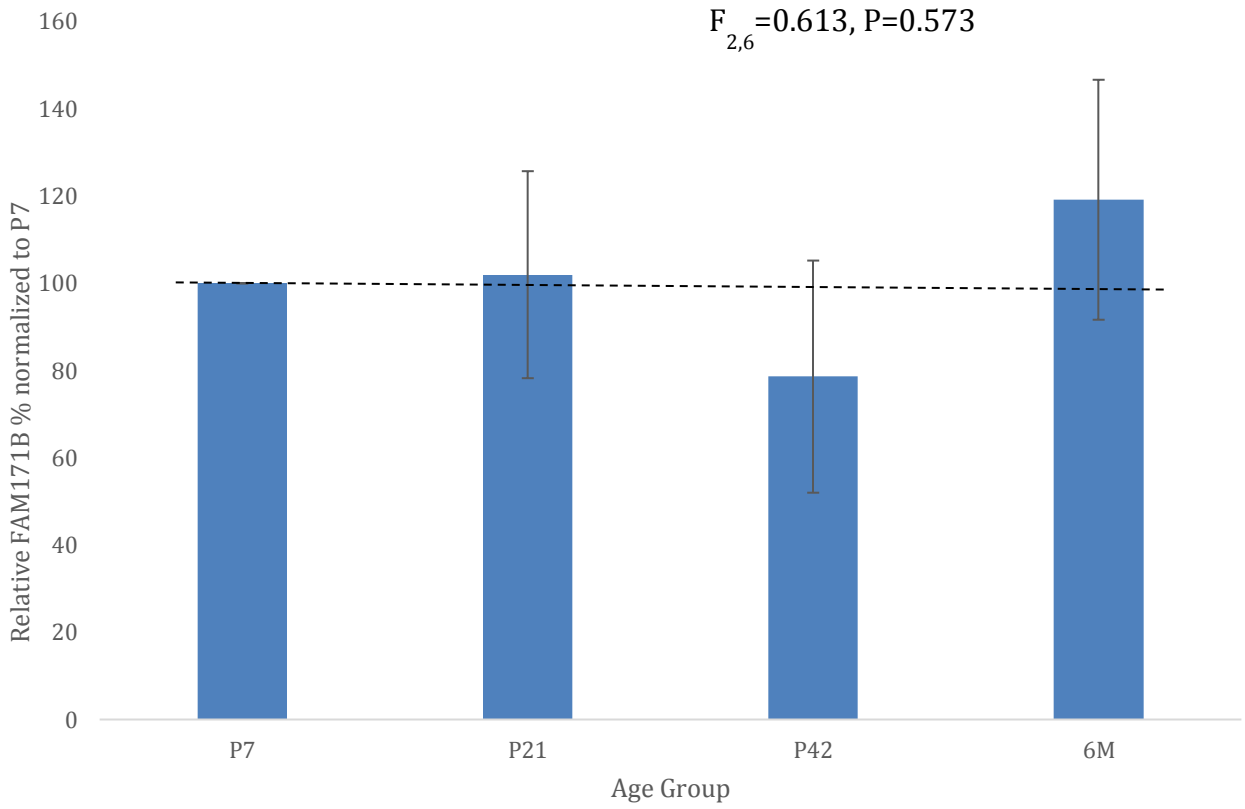

Figure 5. Semi-Quantitative analysis of FAM171B expression in Ammon's Horn region of the hippocampus in mice brains of four different age groups.

Like Ammon's Horn, FAM171B mRNA is also highly expressed in the Dentate Gyrus region of the hippocampus of experimental mice brains across all four age groups (Figure 6). A univariate ANOVA test displayed a P-value of 0.108, which suggests no significant difference in FAM171B expression amongst the different age groups.

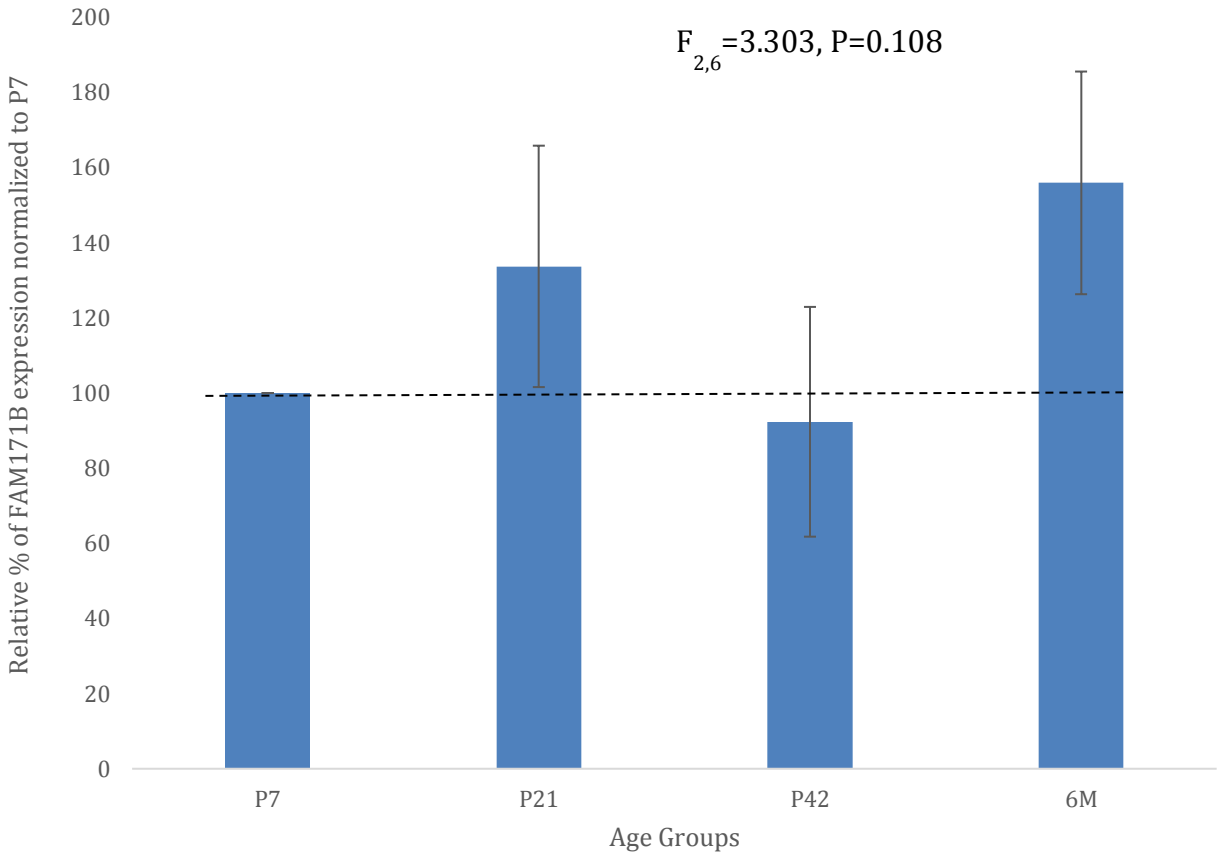

Figure 6. Semi-quantitative analysis of FAM171B expression in the Dentate Gyrus region of the hippocampus of mice brains belonging to different age groups.

3.5 Detection of FAM171B mRNA expression in the Cerebellum

The dark purple staining in Figure 7 and 8 indicates that FAM171B mRNA is expressed in the cerebellum of an adult mouse brain. Upon closer inspection, it is clear that FAM171B mRNA expression is prominent in the Purkinje cell and granular cell layers, and the lowest in the molecular layer (Figure 8).

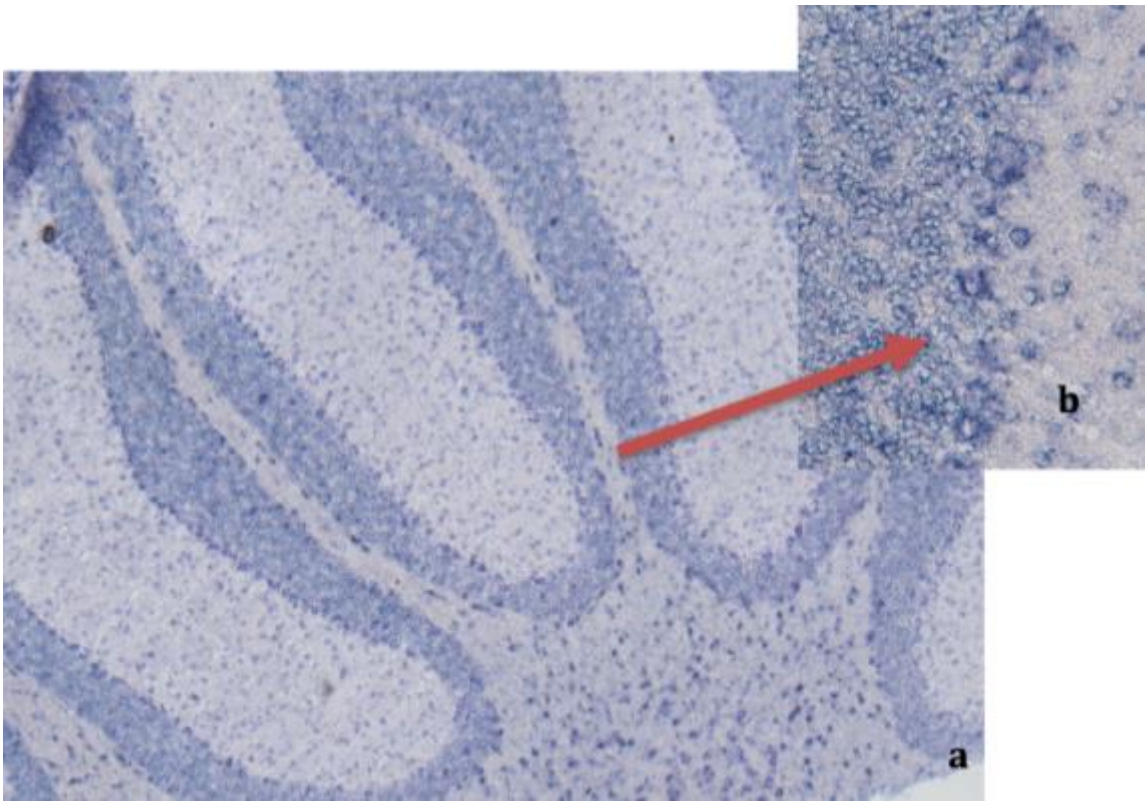

Figure 7. FAM171B mRNA expression in the cerebellum of a 6-month-old mouse brain treated with the experimental probe during *in-situ* hybridization. Images were obtained using the 5X (a) and 40 X (b) objectives of a compound microscope.

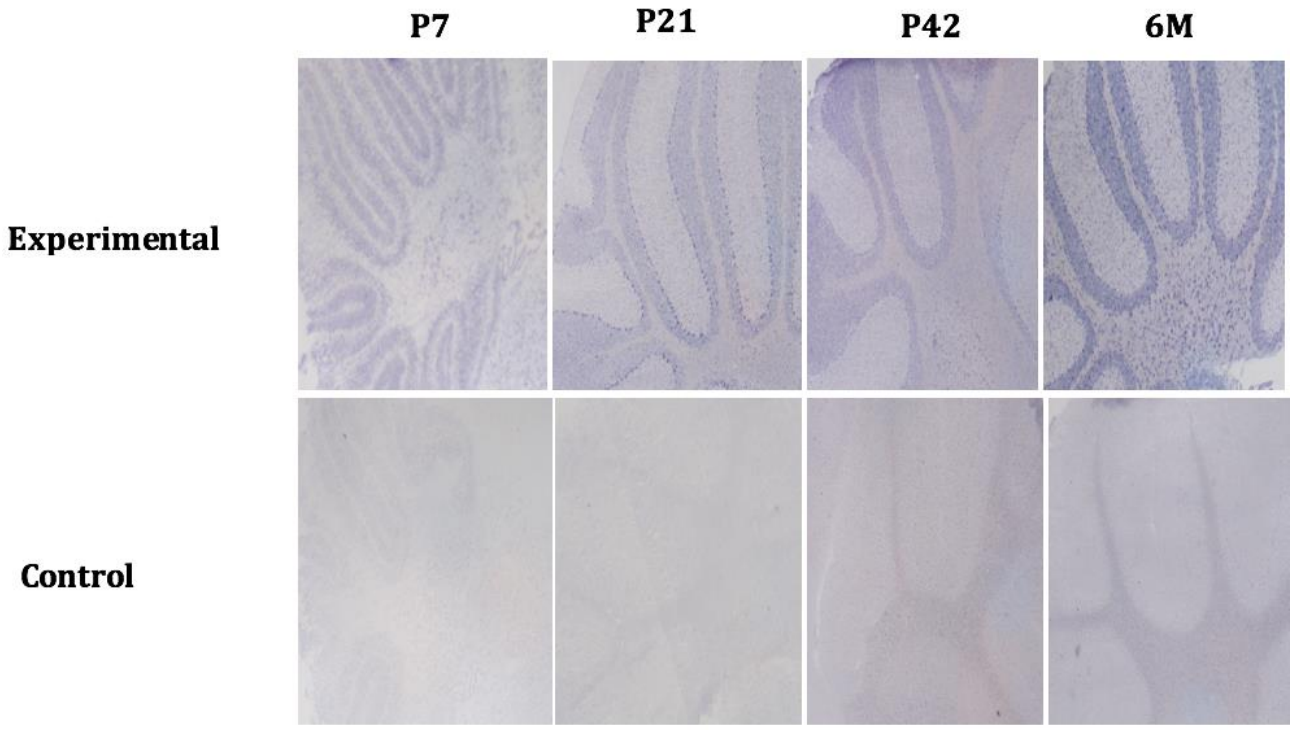

Figure 8. Qualitative analysis of FAM171B expression in cerebellum of mice brains in different age groups treated with experimental probe during *in-situ* hybridization compared to brains treated with control probe. Pictures were taken at 5X objective under compound microscope.

The one-way ANOVA test performed, based on the results of the densitometry (after normalization of the relative percent expression to P7), yielded a P value of P = 0.440, suggesting that FAM171B expression in the cerebellum is not significantly different amongst the 4 different age groups (Figure 9).

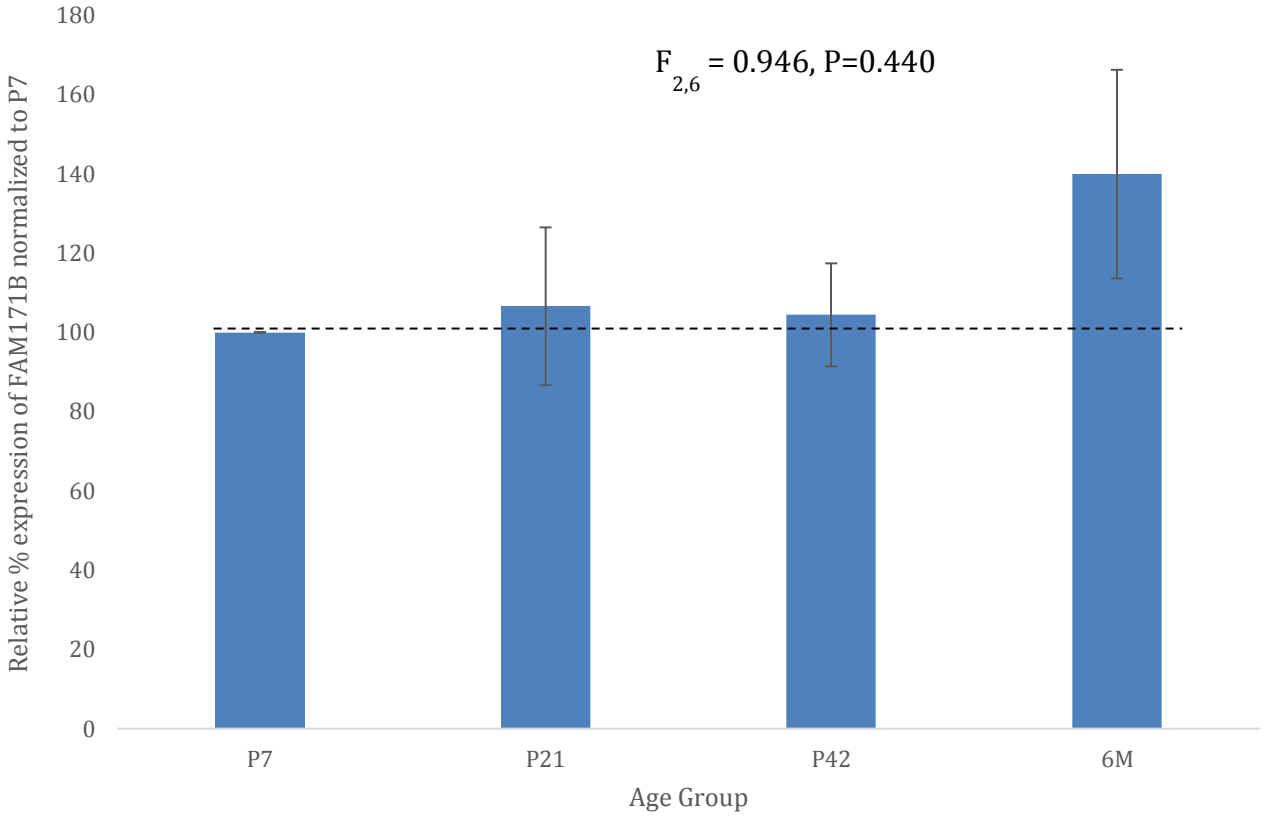

Figure 9. Semi-Quantitative analysis of FAM171B expression on cerebellum in mice brains correspond to different age groups.

Chapter 4

Discussion

FAM171B is an uncharacterized novel protein that contains polyglutamine residues within its amino acid sequence. Given the homology that is shares with proteins that result from mutated genes associated with polyglutamine diseases, FAM171B may also be linked to one or more neurodegenerative polyglutamine (polyQ) diseases. Unfortunately, the location or function of FAM171B is unknown, thus the current study was undertaken to localize the expression of FAM171B in mouse brains. Mice were selected as the model to study FAM171B because genomic sequence data shows that they are known carriers of the FAM171B protein. Moreover, the mouse FAM171B protein has 88% homology with the human FAM171B protein and contains ~7 glutamine repeats (humans have ~ 16 repeats) (39).

The goal of the present study was to localize FAM171B mRNA in mice brains representing 4 different age groups (7-day (P7), 21-day (P21), 42-day (P42) and 6-month old (6M) mice) using *in-situ* hybridization. The data collected indicates that FAM171B mRNA is expressed widely throughout mice brains, regardless of age. However, upon closer examination of each brain section, it was observed that FAM171B expression is more prominent in some brain regions than others (Figure 2, Table 1). Densitometry data indicated there is no significant difference in expression of FAM171B mRNA among age groups, however since densitometry does not precisely measure the level of FAM171B-mRNA expression, we consider this analysis as a semi quantitative analysis.

Qualitative analyses reveal that, of all the brain regions examined, the hippocampus seemed to exhibit the highest level of FAM171B mRNA expression (Table 1). The hippocampus, which is a part of the limbic system and is located in the temporal lobe of the cerebral cortex, is an essential neuronal structure, responsible for playing a key role in consolidation of information from short term memory into long time memory. This structure is also responsible for storing and processing spatial memory that helps with navigation, with the dorsal portion being responsible for learning and forming memories (especially spatial navigation), and the ventral section primarily involved with anxiety associated behavior *(26)*. Many polyglutamine neurodegenerative disorders (*e.g.*, Huntington's disease and Spinocerebellar Ataxia), result in impaired memory and dementia, which may be due to the impaired hippocampus functioning *(38)*. Consequently, the expression of FAM171B mRNA in the hippocampus may suggest that this polyglutamine protein is linked to an underlying neurodegenerative disorder.

A high level of FAM171B mRNA expression was also observed in the cerebellum, the main structural feature of the hind-brain. Qualitative as well as semi-quantitative analyses confirm that FAM171B mRNA is highly expressed in this region of the brain (Figures 7, 8 and 9). The cerebellum is known to play a vital role in attention, language, emotional responses, addiction, pain, working memory, learning and pain *(36)*. Planning and execution of movements or motor control is another important function of the cerebellum in humans *(27)*, although it should be noted that this structure does not initiate movements but is responsible for muscle coordination, precision and precise timing. Structurally, the cerebellum is divided in to three main layers: (1) the molecular, (2)

granular layer and (3) Purkinje cell layers. Of these, the granular layer is the deepest, consisting of densely packed granule cells (the smallest and most numerous neurons in the brain), Golgi cells also known as inhibitory interneurons, unipolar brush cells (excitatory glutamatergic interneurons), and Lugaro cells, sensory interneurons that performs inhibitory functions. The middle narrow layer consists of Purkinje cells, the most distinctive neurons in the brain that are typically recognized by the shape of their dendritic tree that branches profusely. Because Purkinje cells use GABA as neurotransmitter, they exert inhibitory effects on their target(s). Finally, the molecular layer, the outermost layer of the cerebellum, contains basket and stellate cells, both of which are inhibitory interneurons that forms GABAergic synapses with the dendrites of Purkinje cells. Of these 3 layers, qualitative study indicates that the Purkinje cell and granular layers have the most prominent FAM171B mRNA expression in mice brains of all 4 age groups (Figure 9) although this expression is not significantly different among the different age groups. This finding suggests that FAM171B mRNA is present though out brain development of the mice.

In many polyglutamine neurodegenerative disorders the cerebellum is the region of the brain that is impaired. According to Orr *(28)*, Purkinje cells in the cerebellum are severely affected/lost in persons/mice afflicted with spinocerebellar ataxia type 1 (SCA type 1) because of the expanded number (~77) of glutamine residues in the SCA-1 gene product that produces the ataxin-1 protein and, consequently, ataxia. Studies by the above author *(28)* as well as Paulson *et al., (12)* suggest that overexpression of glutamine residues in the SCA3 gene in transgenic mice results rapid cell loss of Purkinje cells, leading to

cerebellar atrophy and ataxia. Moreover, SCA6 (spinocerebellar ataxia type 6), like SCA1, results in severe cell loss in the Purkinje cell layer of the cerebellum. It is very likely that Purkinje cell death in persons with SCA6 is caused by having an excess entry of calcium into the Purkinje cells because of the SCA 6 gene mutation *(28)*. Finally, SCA type 17, Dentatorubral and pallidoluysian atrophy (DRPLA) and Huntington's disease, other polyglutamine diseases caused by having an expanded number of CAG repeats within the mutated gene, results in severe alterations/degeneration in the cerebellum *(16) (17) (29) (37)*. Given that FAM171B protein also contains polyglutamine repeats within its amino acid sequence and is highly expressed in the neuronal layers of the cerebellum (*i.e.*, the Purkinje cell and granule cell layers) throughout development of the brain, this protein may have an underlying relationship with one or more polyglutamine disorders.

Our data also shows that the caudate is another region of the brain that FAM171B mRNA is expressed in. Qualitative analysis of the results of the in-*situ* hybridization indicates that there is moderate expression of FAM171B mRNA in the caudate of mice models representing all 4 of the age groups (Table 1). This region of the brain is known to play a vital role as a feed-back processor where it uses the information from past experience to impact future actions and is also responsible for successful goal directed actions *(30)*. In addition, the striatum, which is composed of both the caudate and the putamen, is responsible for multiple features of cognition including planning, motivation and decision making *(30)*. According to Orr *(28)*, the pathology of Huntington's disease, which impairs most brain regions, results primarily from atrophy of the striatum, leading to a loss of GABA mediated neurons in this region. The same author also reports that neuronal loss,

specifically in striatum, hippocampus, thalamus and cortex, was observed in transgenic mice that expressed full length Huntington's disease cDNA. However, the investigators of the abovementioned study concluded that the severity of Huntington's disease depended upon the length of the polyglutamine tract, the level of the expression of the transgene in mice models and on the protein and the location of polyglutamine tract *(28)*. Given these findings, the observation of FAM171B expression in the caudate in all age groups examined seems fascinating and may suggest a link to an underlying polyglutamine disorder since FAM171B has homology with genes associated with known polyglutamine disorders.

The cerebral cortex, the region of the brain that covers the outer portion of the cerebrum, consists of many folded regions called gyri that increase the surface area of the brain and thus the amount of information that can be processed by the brain *(32)*. This portion of the brain is responsible for many functions including motor movements, planning and organization, intelligence and personality determination, and the processing of language and sensory information *(32)*. Damage to the cortex often results in ataxia, which is characterized by a lack of muscle coordination and balance. Ataxia is the most profound symptom of several types of spinocerebellar ataxias (SCA1, SCA3, SCA6, SCA17 and DRPLA) all of which are classified as polyglutamine neurodegenerative disorders. Although the cortex is affected in these above disorders, the pattern of degeneration and the relationship of cortical degeneration to the clinical symptoms can be difficult to assess due to the complex folding pattern of the cortex *(28)*. Given the association of cortex damage and polyglutamine neurodegenerative disorders, it is not

surprising that in the present study, the cortex was found to express moderate levels of FAM171B mRNA among all age groups examined, with the most prominent expression observed in both the motor and sensory cortices.

The thalamus, the brain's relay center, is located below the corpus callosum of the brain and is considered a part of the forebrain. The thalamus is primarily responsible for conveying information from the sensory receptors to the appropriate region of the brain where the sensory information can be processed *(28)*. As mentioned previously, neuronal loss in thalamus, among other areas of the brain, was observed in transgenic mice expressing full length Huntington's disease cDNA *(28)*. In addition, another study that mapped the MRI data of several Huntington's disease patients revealed that both striatal and thalamic atrophy was prominent among this group *(33)*. Therefore, the observation of moderate levels of FAM171B mRNA in the thalamus of mice models in all 4 age groups is intriguing.

The amygdala, like the thalamus, exhibited moderate expression of FAM171B among all 4 age groups. This portion of the brain is located within the temporal lobes and is a part of a limbic system. The amygdala plays an important role in processing emotions such as anger, fear and pleasure *(34)* and also determines what memories are stored and where in the brain they are stored. Neurodevelopmental disorders such as Williams syndrome, Schizophrenia, anxiety and autism *(34)* are all known to affect the amygdala. PolyQ disorders, in contrast, do not routinely impair this part of the brain, although various levels of Amygdala neurodegeneration have been reported in some Huntington's disease patients *(35)*.

The Corpus callosum, the large bands of white matter that do not have neuronal cell bodies, but connect the left and right hemispheres of the cerebrum, is involved in communication among the different parts of the brain. Qualitative analysis indicates that FAM171B mRNA is not expressed in this region of the brain in any of the 4 age groups examined. These data indicate that FAM171B may only be expressed in neuronal cell bodies (grey matter), but not in the white matter. Furthermore, additional research is needed to determine whether FAM171B is also expressed in glial cells such as astrocytes and microglia.

Chapter 5

Conclusion

FAM171B is a novel protein that possesses a number of polyglutamine tracts (7 in mice vs. 16 in humans) but its function remains relatively unexplored. Sequence analysis of FAM171B, however, reveals that this protein shares some homology with other altered proteins involved in polyglutamine neurodegenerative disorders. Thus, the present study was preformed to examine expression levels of FAM171B mRNA in different regions of mice brains representing 4 different age groups (7-day-, 21-day-, 42-day- and 6-month-old mice). Our findings indicate that although FAM171B mRNA is widely expressed in the brains of mice belonging to each age group, discernable differences in expression were apparent in the hippocampus, cerebellum, cortex, amygdala and thalamus. Our data also indicates that FAM171B mRNA is not expressed in the Corpus callosum, the large tracts of white matter between the left and right cerebral hemispheres, suggesting that FAM171B is most likely expressed only in neuronal cell bodies. In addition, the results of this study indicate that FAM171B mRNA expression is consistent among the 4 different age groups, implying that FAM171B is probably present in mice brains throughout their development. Given that FAM171B, like other polyQ proteins, contains a polyglutamine stretch and has high expression levels in the aforementioned brain regions, this protein may have an underlying role in one or more polyglutamine neurodegenerative disorders that have yet to be molecularly characterized. Future work includes examining FAM171B protein levels and expression patterns via immunohistochemistry, and investigating the intracellular expression of FAM171B using immunofluorescence techniques.

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Appendix

7.1 pGEM-T Easy vector properties

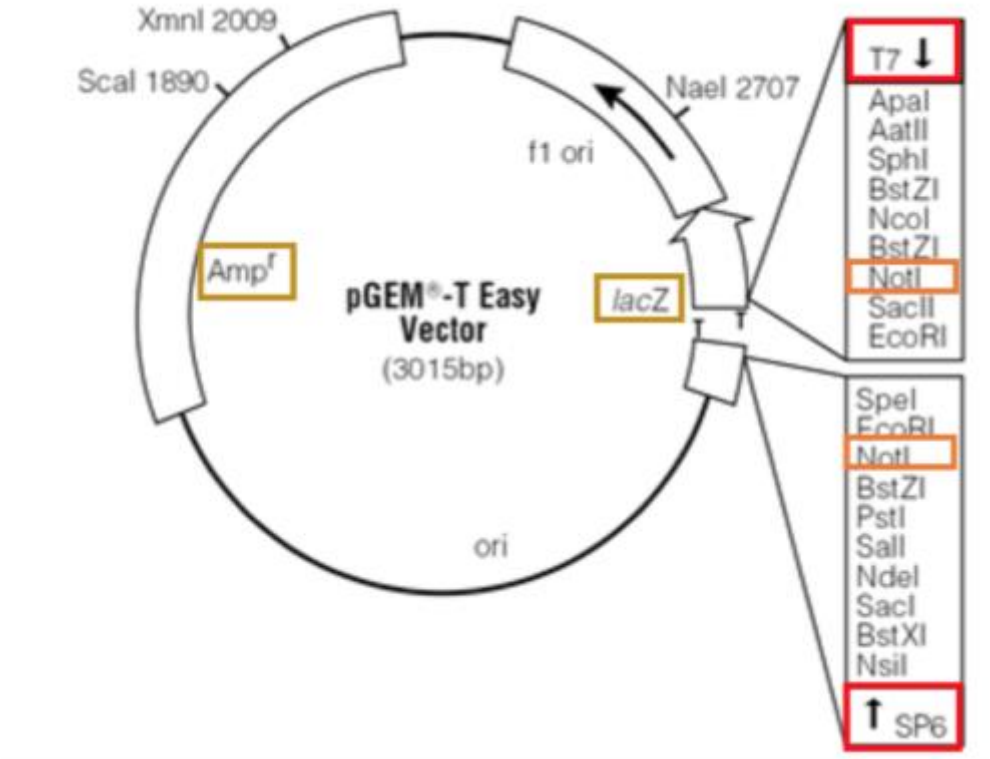

Figure 1: pGEM-T Easy vector map (www.promega.com)

7.2 Transformation of Vector to JM109 Bacteria

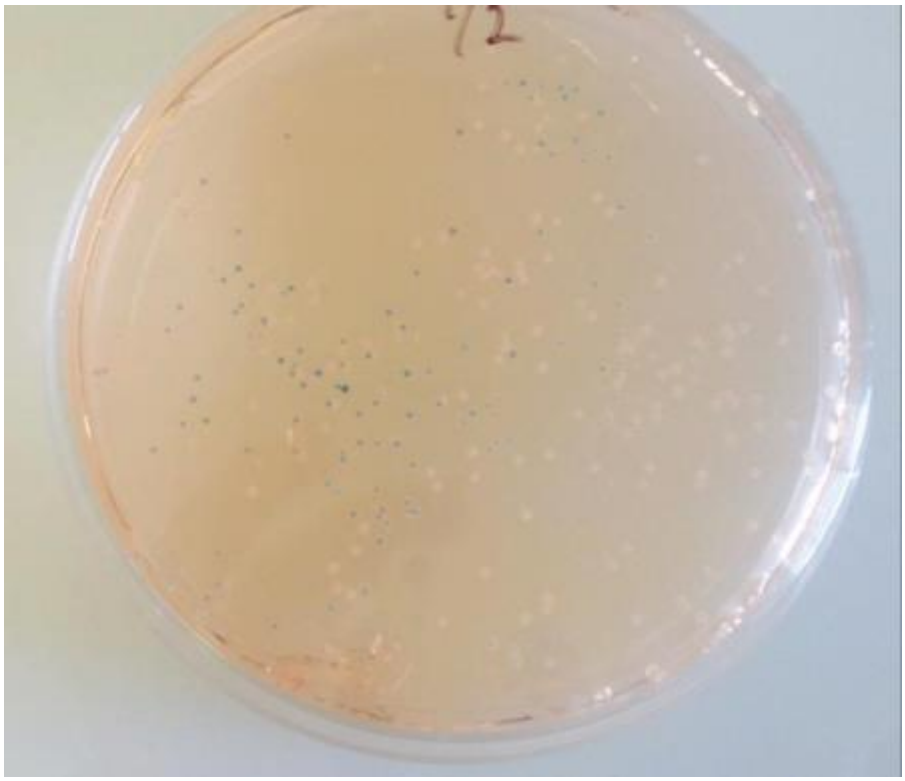

Figure 2. Transformation of pGEM-T Easy vector to JM109 bacteria. Blue colonies indicate transformed vectors without the FAM171B insert. White colonies Indicate transformed vectors with FAM171B insert.

Figure 2 contains JM109 bacterial colonies after transformed with pGEMT-Easy vector. These bacteria were grown in a media with ampicillin. Therefore, all these bacteria that grew in this specific media transformed the pGEM-T Easy vector because vector it-self corresponded with ampicillin resistance. Since X-gal was also added to this media, it can recognize Lac-Z marker on the vector and change the color of the bacteria to blue color, if that marker was not interrupted by an insert in between. Therefore, bacterial colonies

that were colored in blue in this figure contains the pGEM-T Easy vector but not the FAM171B insert and white colonies contain the pGEM-T Easy vector with the FAM171B fragment.

7.3 Confirmation of Restriction Digest with NotI

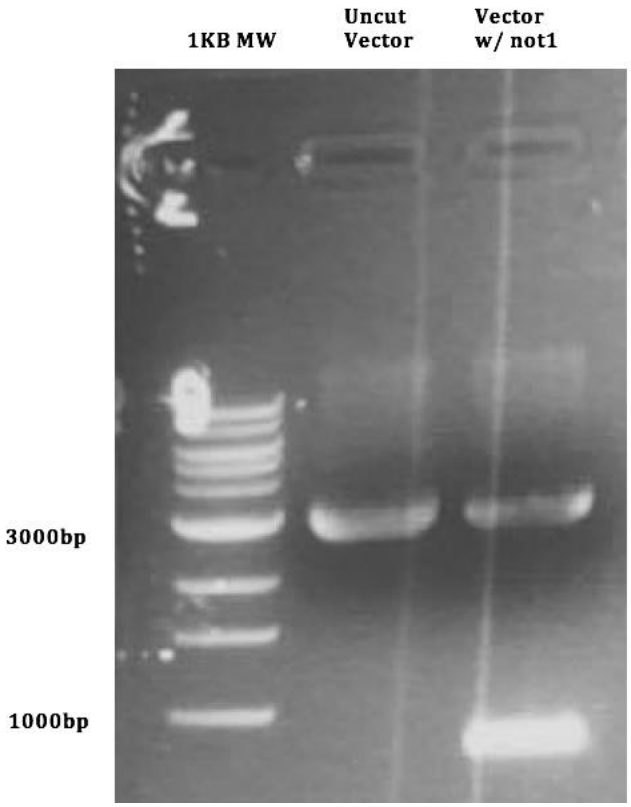

Figure 3. Gel electrophoresis analysis confirming restriction digest of the vector with NotI enzyme. 1kb DNA ladder on the first lane. Uncut pGEM-T Easy vector with FAM171B on the second lane . pGEM-T Easy vector(3000bp) and FAM171B fragment (800bp) after restriction digest with NotI on the third lane.

Figure 3 indicates gel electrophoresis analysis of restriction digest of the pGEM-T Easy vector using NotI restriction enzyme. This procedure is only conducted to confirm to see if the vector contains the desired FAM171B fragment. NotI enzyme cuts at both SP6 and T7 promotor regions of the vector allowing to isolate the FAM171B fragment. Therefore, when pGEM-T Easy vector cut with NotI, we should be able to see two bands on the gel, one band corresponds to the size of the vector which is 3000bp and the other band corresponds to the size of the FAM171B, which is 800bp. Looking at two bands appeared on lane C of the figure 3 and by comparing to the size of the bands to the DNA ladder confirms that FAM171B is in the vector. This data was also confirmed by DNA sequencing.

7.5 Primers used for Fam171B amplification

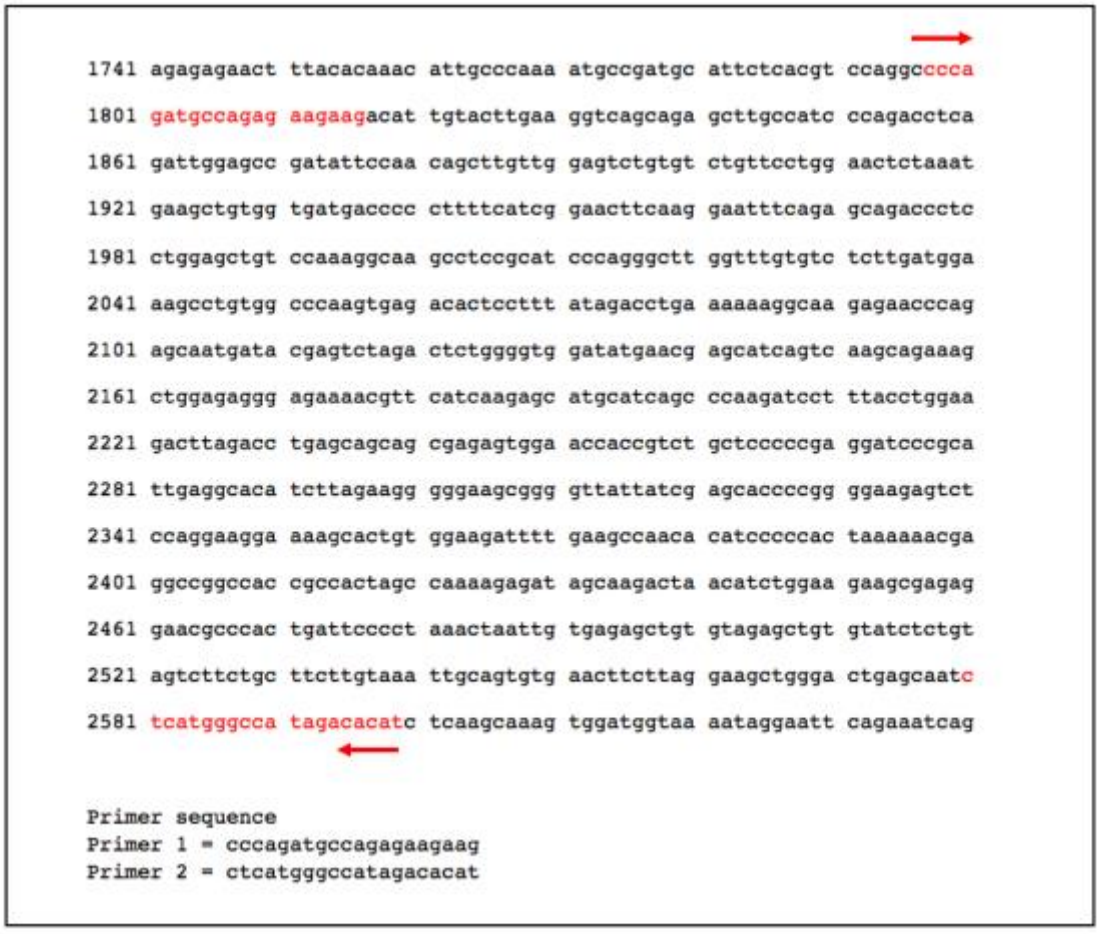

Figure 4: Primers used in FAM171B amplification

7.4 DNA sequencing data for FAM171B

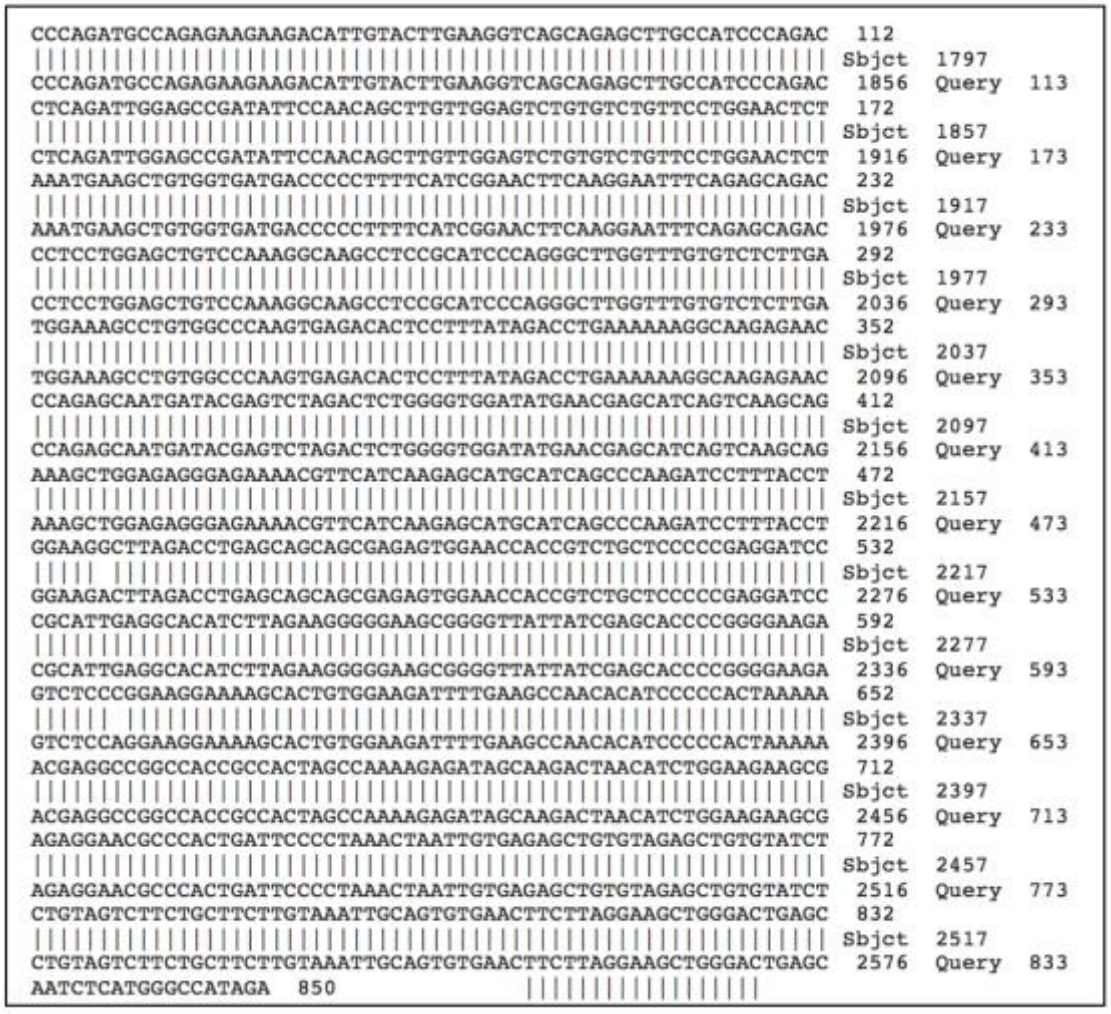

Figure 5. DNA sequencing data for FAM171B on the SP6 Probe

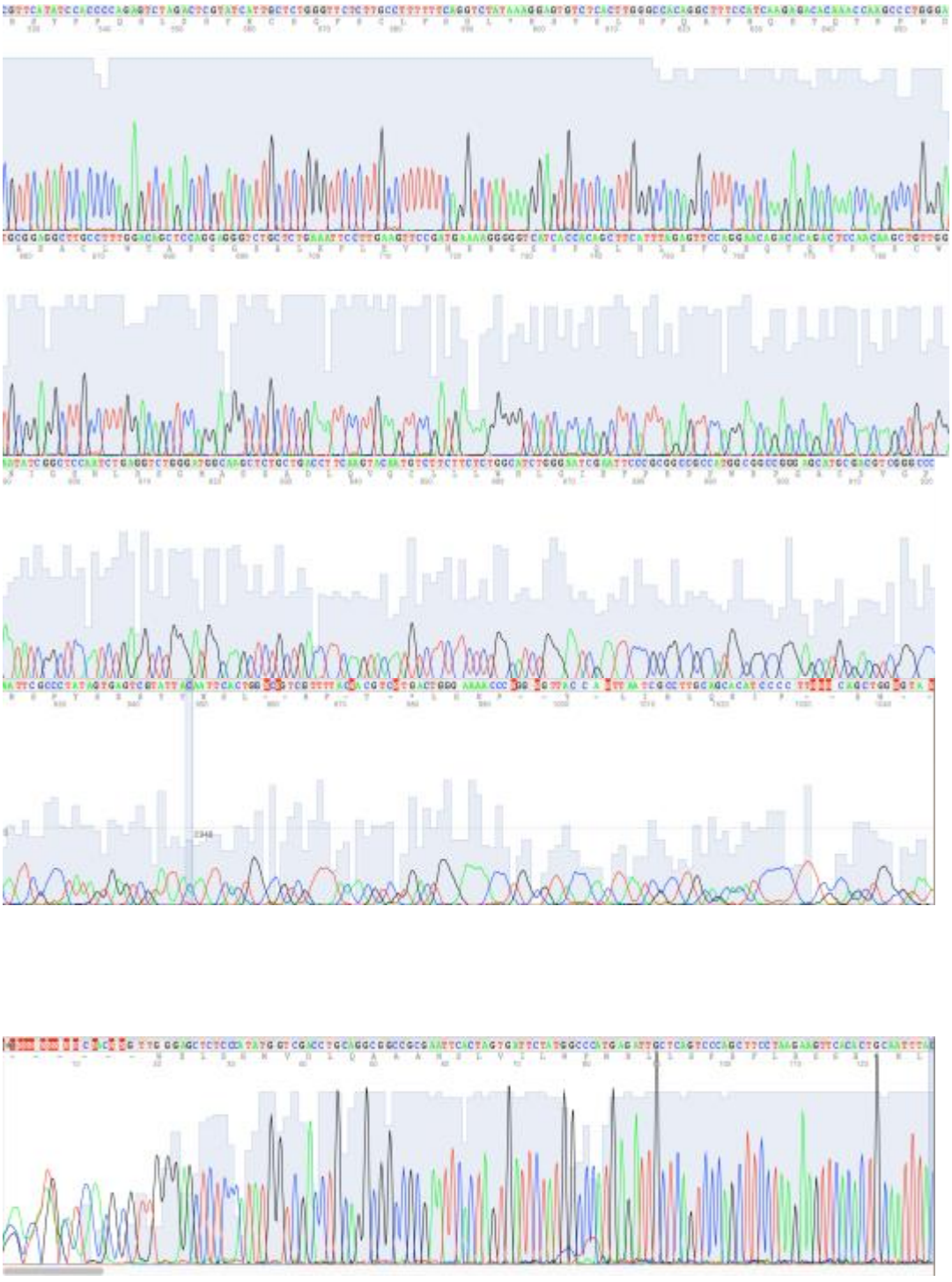

Figure 6. DNA Sequencing data for SP6 probe

7.5 Restriction Digest with ApaI and SalI

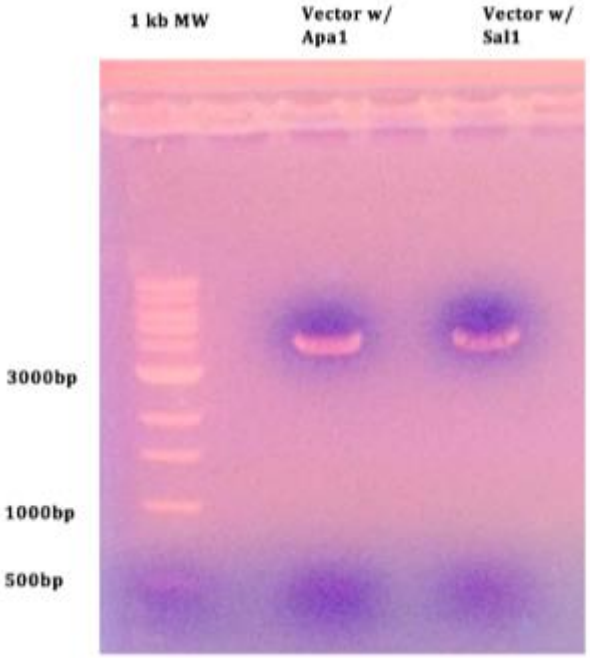

Figure 7. Gel electrophoresis analysis of the Linearization of pGEM-T Easy vector using ApaI and SalI restriction enzymes. 1kb DNA ladder on the first lane. pGEM-T Easy vector cut with ApaI on the second lane . pGEM-T Easy vector cut with SalI on the third lane.

Figure 7 indicates the gel electrophoresis of restriction digest of pGEM-T Easy vector using ApaI and SalI. ApaI restriction enzyme cuts at the sp6 promotor region of the vector and salI restriction enzyme cuts at the T7 promotor regions of the vector. Linearization is important because when we later conduct the in-vitro transcription of FAM171B, linearization helps to avoid transcription of mRNA in a circular manner. ApaI and SalI restriction enzymes helps to make control and experimental mRNA probes. Thus, we know the direction of Fam171B after the DNA sequencing, vector cut with ApaI identified as the experimental FAM171B contained plasmid and SalI, identified as the control FAM171B contained vector.

7.6 Confirmation of FAM171B mRNA after the transcription reaction

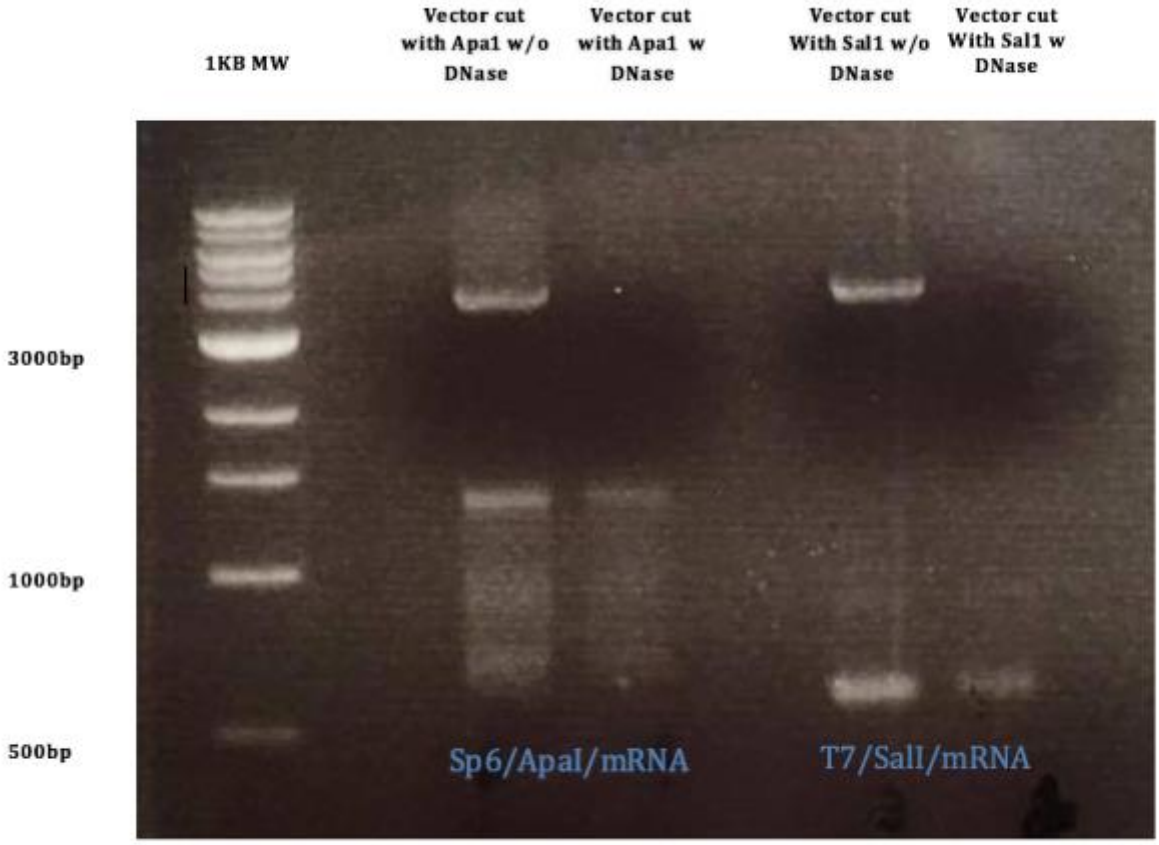

Figure 8. Gel electrophoresis analysis of *In-vitro* transcription of FAM171B mRNA. 1kb DNA ladder (A). Experimental probe sp6 promotor region cut with ApaI without DNase (B). Experimental probe sp6 promotor region cut with ApaI and DNase added (C). Control probe T7 promotor region cut with SalI without DNase (D). Control probe T7 promotor region cut with SalI and DNase added (E).

Figure 8 illustrates the *in-vitro* transcription of FAM171B using T7 and Sp6 promotor regions. Experimental and control FAM171B mRNA probes were prepared using

ApaI and SalI restriction enzyme and by using SP6 and T7 promotor regions of the vectors. These experimental and control probes were designed based on the orientation of FAM171B DNA on mouse tissue from DNA sequencing data. Experimental FAM171B mRNA probe will have the complementary mRNA sequence to the mRNA present in mouse tissues whereas Control FAM171B mRNA probe will have the same sequence to the mRNA present in the mouse tissue. In lane B and D, DNA bands corresponds to the vector size present in both experimental and control probes due to not adding DNase. In lane C and E DNA bands corresponds to the size of vector are not present in both control and experimental probes in the gel due to addition of DNase. In lane C and E only the mRNA bands can be visualized. Since these mRNA was single stranded, mRNA tends to run in the gel in a distorted manner resulted creating various bands. FAM171B mRNA experimental and control mRNA probes were present in figure 5 between 1000bp and 500bp band on the 1kb size standard. During this procedure, non-canonical DIG labeled bases and DNase were added in to the reaction mixture.