BIOCHEMICAL CHARACTERISATION OF CG15111 & CG1309

_ FLY ORTHOLOGUE OF ABHD12 & ABHD16A

A Thesis

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by

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From May 2022 to Mar 2023

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CERTIFICATE

This is to certify that this dissertation entitled **Biochemical Characterization of fly genes CG15111 and CG1309, the fly orthologue of mammalian ABHD12 AND ABHD16A** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Rohith C S** at Indian Institute of Science Education and Research under the **supervision of Dr. Siddhesh S Kamat, Associate Professor**, Department of Biology, during the academic year 2022-2023.

Dr. Siddhesh S Kamat

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Dr. Girish S Ratnaparkhi

This thesis is dedicated to

my Acha, Amma, Kochu and that one special person in my life.

DECLARATION

I hereby declare that the matter embodied in the report entitled **Biochemical Characterization of fly genes CG15111 and CG1309, the fly orthologue of mammalian ABHD12 AND ABHD16A** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Siddhesh S Kamat and the same has not been submitted elsewhere for any other degree.

Rohith C S

Date: 10/04/2023

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ABSTRACT

PHARC is an autosomal recessive neurological disorder caused a null mutation in ABHD12 gene. The murine model for PHARC is kind of characterized but the other model organism like *Drosophila* is not well characterized. We here try to characterize the fly genes CG15111 and CG1309, the orthologues of ABHD12 and ABHD16A as the deregulation of the amount of lyso-PS will lead to PHARC pathology and these 2 genes regulates the amount of lyso-PS in mammalian brain. Here we have done initial step to characterize these genes and make a fly model for PHARC pathology and finding their effects in the fly immunity.

CONTRIBUTIONS

Contributor name	Contributor role
Dr. Siddhesh S Kamat	Conceptualization Ideas
Kumar Kundan, Rohith C S	Methodology
Rohith C S, Kumar Kundan	Software
Rohith C S	Validation
Rohith C S, Kumar Kundan	Formal analysis
Rohith C S	Investigation
Dr. Siddhesh S Kamat, Dr. Girish Ratnaparkhi	Resources
Rohith C S	Data Curation
Rohith C S, Kumar Kundan	Writing - original draft preparation
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-	Visualization
Dr. Siddhesh S Kamat	Supervision
Dr. Siddhesh S Kamat	Project administration
Dr. Siddhesh S Kamat	Funding acquisition

1. INTRODUCTION

1.1 Phospholipase

The group of enzymes which cleaves phospholipids, a major class of lipids found in biological systems, at specific positions are known as *Phospholipases*. The general structure of a phospholipid is a glycerol backbone which has two fatty acids in one end (non-polar tail group) and the other end has an esterified phosphoric acid and an organic alcohol group (polar head group). There are 4 sites where phospholipids are cleaved by phospholipases and based on that phospholipids can be classified into 5 different groups. Phospholipase A1 (PLA1), Phospholipase A2 (PLA2), Phospholipase B (PLB), Phospholipase C (PLC) and Phospholipase D (PLD). PLA1 cleaves the phospholipid at the SN1 position where the end products are lyso-phospholipid and fatty acid Major substrates for PLA1 are phosphatidylcholine (PC), Digalactosyldiacylglycerol (DGDG), Phosphatidic acid (PA), Monogalactosyldiacylglycerol (MGDG), Phosphatidylserine (PS) etc. Similarly, PLA2 cleaves at the SN2 position to remove the fatty acid and the major substrates are PE, PC, Glycerophospholipids etc. The special feature of PLB is that it can cleave both SN1 and SN2 position and substrates includes Lysolecithin, Glycerophospholipids, PC. The phosphodiester bond between the glycerol backbone and the phosphate group in the phospholipids are cleaved by PLC. The final group PLD removes the head group from the phospholipids and produce Phosphatidic acid. Phospholipases are one of the most important group of enzymes that have impact on various areas in our body like signal pathways, membrane remodeling and inflammatory responses etc. The membrane phospholipids are hydrolyzed by PLA2 to release Arachidonic acid which can be metabolized to various signaling molecules in inflammation (Dennis & Norris, 2015). Membrane remodeling is essential for processes like endocytosis, exocytosis, and membrane fusion, PLD plays a important role in regulating this as it cleaves PC to release phosphatidic acid which is the signalling molecule that regulates the curvature and fusion of membranes(Exton, 2002).Malfunction of these enzymes will lead to severe diseases. The study on phospholipases has a high therapeutic value as inhibitors for PLA2 is used as anti- inflammatory agents (Dennis & Norris, 2015) and PLD is the potential target for cancer treatment (Foster & Xu, 2003).

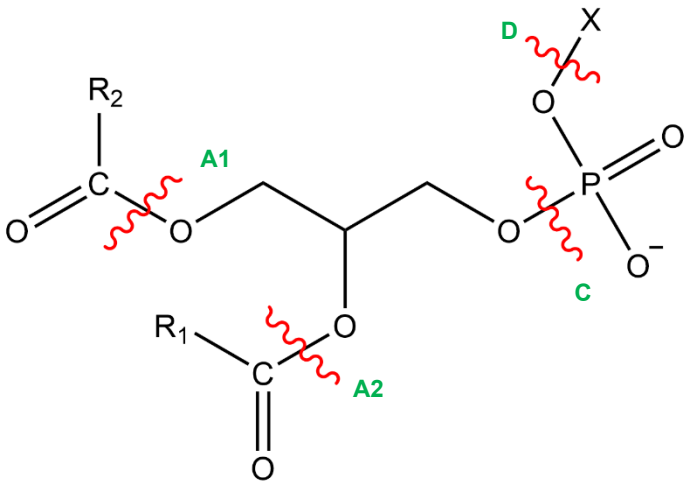


Figure 1.1 Cleavage sites of different Phospholipases

1.2 Phosphatidylserine Lipase and Lyso-phosphatidylserine Lipase

Phosphatidylserine lipases (PS lipase) are a class of which cleaves phosphatidylserine (PS). It hydrolyses the ester bond and release lyso-phosphatidylserine and free fatty acid. Lyso-PS has a major role in signalling pathways. It is highly enriched in immune cells and rising the fact that they have been involved in auto immune disorders(Chu et al., 2013; Szymański et al., 2014; Kamat et al., 2015). It is seen that PS lipase has a role in membrane remodelling. PS lipase seem to regulate cell proliferation and apoptosis, it is seen that inhibition of PS lipase has lead to the cell cycle arrest and apoptosis (Bougnoux et al., 2009). PS lipases are also involved in maintaining the homeostasis of membrane lipids. The study on yeasts found that the deficiency of PS lipases will change the organisation and composition the membrane lipids and will lead to cell death (Adeyo et al., 2011).

In the mammalian brain system *Alpha Beta Hydrolase Domain Containing Protein 16A* also known as ABHD16A act as the PS lipase and it also has acylglycerol lipase activity. It comes under the category serine hydrolase, where the active site contains a serine residue. ABHD16A has a major role in lipid metabolism and signal transduction (Blankman & Cravatt, 2013). Its involved in the metabolism of endocannabinoids, signalling molecules that are involved in processes like pain sensation, appetite, inflammation and mood (Bisogno & Maccarrone, 2014). ABHD16A is mainly expressed in cerebellum and very poorly expressed in the other areas of the brain and it is located in the endoplasmic reticulum (ER). Mutation will lead to Hereditary spastic paraplegia (HSP), a neuro degenerative disorder.

Lyso-PS lipases are the enzymes will cleave the lyso-PS and release free fatty acid and glycerophosphoserine. Lyso PS has a lot of roles in our body so by regulating lyso-PS, lyso-PS lipase also takes part in these functions apart from its independent ones. The major functions include the maintaining of cellular homeostasis, inflammation related regulations, regulation of insulin signalling(Yea et al., 2009) and lipid metabolism.

In the mammalian brain ABHD12 act as a lyso-PS lipase, a serine hydrolase. ABHD12 has a role in lipid metabolism, signal transduction, protein degradation. It also regulates cleavage of endocannabinoid neurotransmitter 2-arachidonylglycerol (2-AG) in the CNS (Savinainen et al., 2012). ABHD12 is expressed ubiquitously all over in brain and it is an ER membrane associated protein. ABHD12 usually prefers long chain lipids and the activity of ABHD12 losses upon deglycosylation (Joshi et al., 2018a). Apart from these ABHD12 also act as a oxidised PS lipase and it is to be noted that oxidised PS is a potent apoptotic signal as it has a flipped orientation, so ABHD12 has a major role in apoptosis (Kelkar et al., 2019). A null mutation in ABHD12 will lead to the autosomal recessive neurological disorder PHARC.

1.3 PHARC

A autosomal recessive neurological disorder that is caused by a null mutation in mammalian gene ABHD12 (Fiskerstrand et al., 2010). PHARC is attributed by Polyneuropathy, Hearing loss, Ataxia, Retinitis pigmentosa, Cataract, represents a rare genetic condition that has a big impact on a person's quality of life. PHARC disorder affects both the nervous system and sensory perception, posing a number of difficulties for those who are affected by it. Although the full expression of this progressive neurological condition may not happen until age, indications and symptoms usually start in childhood (Dias Bastos et al., 2021). The early symptoms include difficult in hearing of high frequency sounds and in noisy environments, loss of body coordination like walking and body balance, Peripheral neuropathy and difficulty seeing in low light etc. And these can gradually progress and cause complete damage like loss of vision, difficulty in walking, numbness and weakness in limbs, and cataract etc. Patients with PHARC are frequently misdiagnosed because the symptoms resemble those of other diseases, such as Charcot-Marie-Tooth disease, Refsum disease, Retinitis Pigmentosa, and mitochondrial diseases (CMT).

A murine model of PHARC enables researchers to study the disease progression and its impact on various physiological systems, shedding light on potential therapeutic approaches. This model not only contributes to our understanding of PHARC disorder's mechanisms but also paves the way for the development of novel treatment strategies to help improve the lives of those affected by this rare condition. Together using murine model and the tissue culture researchers are trying to unfold the effect of ABHD12 in PHARC disorder. Deregulation of lyso-PS in the brain is the major cause for PHARC. Studies have shown that ABHD12 and ABHD16A act as lyso-PS lipase and PS lipase respectively (Blankman et al., 2013; Kamat et al., 2015). Thus ABHD12 and ABHD16A together regulates the amount of lyso-PS in our brain (Kamat et al., 2015). The ABHD12-/- mice showed the similar phenotype as PHARC pathology. Preference of ABHD12 is also found to be very long chain lipids (Joshi et al., 2018b). The oxidized PS has a filliped membrane orientation which act as a potent apoptotic signal and oxidized PS also triggers release of cytokines which cause neuroinflammation, this will lead to PHARC pathology.

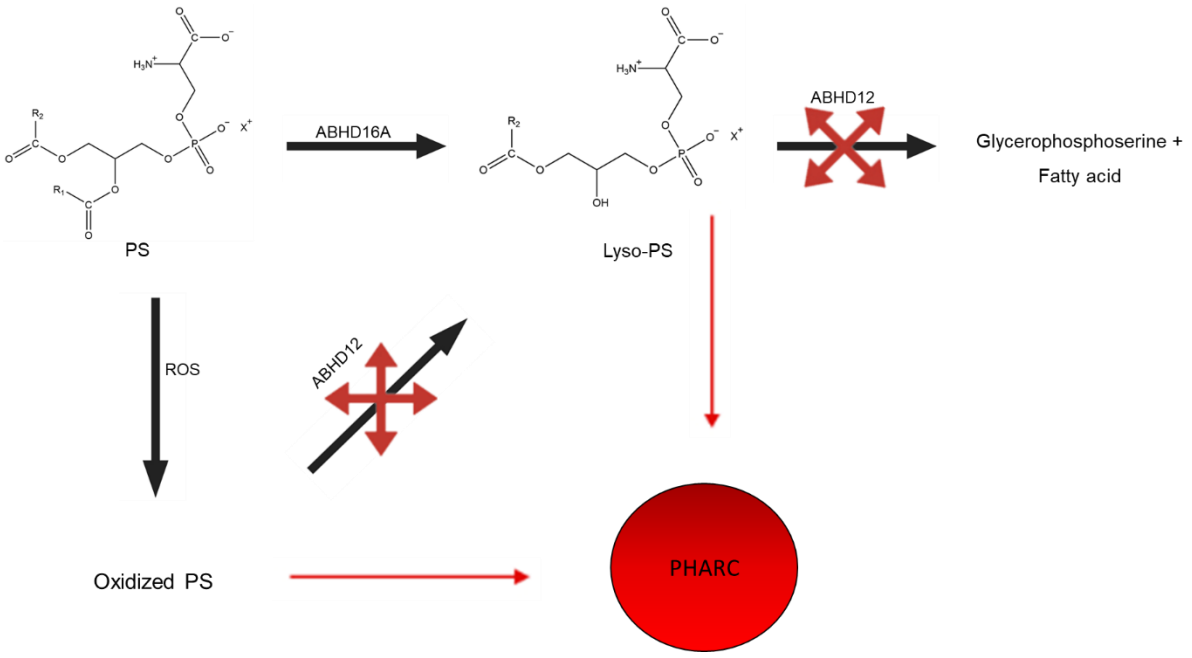


Figure 1.2 Schematic diagram of ABHD12 mutation leading to PHARC pathology

1.4 Fly Model

The fruit fly, or *Drosophila melanogaster*, is a impactful model organism in the area of genetic studies. The primary invertebrate model used now to study developmental genetics is *Drosophila*. *Drosophila* is an ideal model organism for a number of reasons.

First off, the female fly can lay up hundreds of eggs in her lifetime, which ensures a continuous distribution of Drosophila for research. Second, they have a short life cycle thus making multiple generations of fly is rapid and easy. *Drosophila* being very small and easy to maintain thus require minimal culturing requirement. This implies that numerous *Drosophila* can be raised and studied in a modest laboratory without sufficient resources in terms of time, space, or funding. Since Drosophila's entire genome has been sequenced and annotated, it can be used as a valuable tool for genetics.

The fly model for PHARC hasn’t generated yet. In the fly system CG15111 and CG1309 are orthologous to mammalian ABHD12 and ABHD16A and they haven’t characterized yet. *Drosophila* being a short life cycle organism compared to the murine model the researches can be done in more rapid manner if we could make *Drosophila* model for the PHARC. IT took a lot of years of hard work to unfold the PHARC to till what we know and we need to discover a lot of things regarding it, so if we could use Drosophila as a model organism, we can discover more in quick way so that we can give the world a new insight to fight against genetic diseases. Thus, generating a fly model for PHARC using these genes will build a new way to approach the PHARC in more rapid way.

2. MATERIALS AND METHODS

Western Blot

For checking the expression samples were initially ran on a standard 10% SDS gel (10% resolving and 6% stacking) with Bio-Rad pre-stained protein ladder and then transferred to a polyvinylidene difluoride (PVDF) membrane using tank transfer system with standard 1x Transfer buffer at 4°C with a constant voltage supply of 80V for 4 hours. After completion of the transfer, checked the extend of transfer with ponceau red stain and washed off the stain using 0.1% TBST (Tris-buffered saline with 0.1% (v/v) Tween©20). Blot membrane was then blocked using 5%(w/v) milk for 1 hour followed by primary antibody probing for 12-16 hours at 4°C. After the probing, wash the membrane with 0.1% TBST (3 times) then incubated with appropriate secondary antibody for 1hour at room temperature. On completion of the incubation repeated the 0.1% TBST washed 3 time then developed the membrane using Immobilon® Western Chemiluminescent HRP substrate and imaged in Gbox.

Primary Antibodies	Secondary Antibodies
ABHD12	α Rabbit HRP
ABHD16A	α Rabbit HRP
Monoclonal Anti His Tag Mouse (SC-8036)	α Mouse HRP
Anti-His Polyclonal	α Rabbit HRP
Anti-FLAG	-

Table 2.1 List of antibodies_ *List of primary and secondary antibodies used in western blotting*

SLiCE Cloning

Seamless Ligation Cloning Extract is the cloning technique used for generating desired plasmid. Extract was already made from PPY cells (*E. coli* strain) and was kept at -80°C in 1.5mL microcentrifuge tube. The vector used is pRM (3849 bp) and gene of interest are CG15111 (1233 bp) and CG1309 (1572 bp). To clone CG15111 and CG1309 into pRM vector, Primers were designed using snap-gene tool and having 20 nucleotide of homologs sequence. Both CG15111 (Primer 1 & 2) and CG1309 (Primer 3 & 4) was amplified from adult cDNA library using respective primer mentioned in Table 2.2. PCR generated vector is amplified using primers

(Primer 9 and 10) that have common end as insert. The quantity of each reagent to choose from the polymerase protocol manual. The PCR conditions for *Pfu* DNA polymerase by Thermo Fisher Scientific for amplifying gene and vector is mentioned in the Table 2.3.

Sl NO	Primer Name	Primer sequence (5' – 3')
1	CG15111 FP	CATCACCACCACCATCACATGTACGAGATTCATAATTGCCTA
2	CG15111 RP	TCGACTCTAGAGGATCCCTAGTATACGGCGTCACGGTAGT
3	CG1309 FP	CATCACCACCACCATCACATGAGTTTCCTAAATTACGTGTTTGGC
4	CG1309 RP	AGCCACCAATGGCCCAGCCATACAA
5	CG15111SA FP	TCTGGGGCCATGCCCTTGGCACCG
6	CG15111SA RP	CGGTGCCAAGGGCATGGCCCCAGAC
7	CG1309SA FP	GTATGGCTGGGCCATTGGTGGCTTTA
8	CG1309SA RP	AGCCACCAATGGCCCAGCCATACAA
9	C pRM FP	GGATCCTCTAGAGTCGACCT
10	C pRM RP	GTGATGGTGGTGGTGATGCATGGTACCGAGCTCGAATTC
11	pRM Seq FP	AGCATCTGGCCAATGTGC
12	pRM Seq RP	ATTTTTCCATATTTTTCATGGAAAGTTTTA
13	CG1309 FP	CACCATCACCATCACCATTAAGGATCCTCTAGAGTCGACC
14	CG1309 RP	ATGGTGATGGTGATGGTGGGTGAACACAAATCCCTGC
15	CG15111 FP	GAATTCGAGCTCGGTACCATGTACGAGATTCATAATTGCCTAAG
16	CG15111 RP	TCATCATCATCCTTGTAATCGTATACGGCGTCACGGTAGTT
17	CG1309 FP	GAATTCGAGCTCGGTACCATGAGTTTCCTAAATTACGTGTTTG
18	CG1309 RP	CTTATCATCATCATCCTTGTAATCGGTGAACACAAATCCCTGCTCAGT
19	FLAG RP	CAGGTCGACTCTAGAGGATCCCTACTTATCATCATCATCCTTGTAATC
20	C pRM FP	GGATCCTCTAGAGTCGACCT
21	C pRM RP	CACGTAATTTAGGAAACTCATGGTACCGAGCTCGAATTC
22	pRM Seq FP	AGCATCTGGCCAATGTGC
23	pRM Seq RP	ATTTTTCCATATTTTTCATGGAAAGTTTTA

FP	Forward Primer
RP	Reverse Primer

N terminal 6x His tag primers	C terminal 6x His tag primers	C terminal FLAG tag primers

Table 2.2 List of Primers_ *List of primers used for cloning for addition of tags and making homologous ends. The primers with SA in name are used for PCR based mutagenesis for specific gene of interest and the base pairs which makes serine to alanine mutation is labelled in red colour. The colour code is used to identify which primer is used for addition of tag*

	Initial denaturing	Denaturing	Annealing	Extension	Final extension	Hold
Time	2:00 Min	00:15 Min	00:20 Min	1.5 – 4 Min	05:00 Min	
Temperature	95°C	95°C	52 - 65°C	72°C	72°C	4°C

Table 2.3 PCR Settings_ *PCR temperature and time settings at each stage for Pfu DNA polymerase*

PCR based mutagenesis carried out using the primers 5 & 6 for CG15111 and primers 7 & 8 for CG1309 with their respective forward and reverse primers. Two set of PCR reactions are carried out for this mutagenesis. The reactions are primers are listed below (Table 2.4).

	CG15111			CG1309		
	Template	FP	RP	Template	FP	RP
PCR Reaction 1	CG15111	Primer 1	Primer 6	CG1309	Primer 3	Primer 8
	CG15111	Primer 5	Primer 2	CG1309	Primer 7	Primer 4
PCR Reaction 2	Mixture of PCR 1 Products	Primer 1	Primer 2	Mixture of PCR 1 Products	Primer 3	Primer 4

Table 2.3 Mutagenesis Reaction Reactants_ *Set of Primers and Templates used for each reaction for PCR based mutagenesis.*

Component	Volume for 50µL reaction	Final Concentration
10X AccuPrime™ *Pfx* reaction mix	5µL	1x
Primer mix (10µM each)	1.5µL	3µM each
Template DNA	1µL	100ng<
AccuPrime™ *Pfx* DNA polymerase	1µL	
Nuclease free water (NFW)	41.5µL	-

Table 2.5 Mutagenesis Reaction set up_ *Quantity of each reagent used for PCR reactions*

As mentioned above two parallel PCR reactions are carried out with the combination of primers mentioned in 'PCR Reaction 1' (Table 2.4). The PCR products will have a common end where the point mutation occurred. Mix the PCR products together and use that as template for next PCR reaction and the primers used are mentioned in Table (2.4). The PCR product will have the desired serine to alanine mutation. After

agarose gel electrophoresis purified the DNA by gel extraction using QIAGEN Gel extraction kit and quantified using Nano-drop and samples are kept at -20°C.

To do SLiCE reaction or cloning thawed PPY cell extract in ice for 10 minutes followed by addition of vector (100 ng) and gene of interest with homologous ends (400 ng) in the hood. Vertex 1-2 times and keep in the ice for 20 minutes after that gave heat shock at 42°C for 90 seconds then ice shock for 5 minutes. Added 800µL of fresh autoclaved Luria broth (LB) to the extract and incubated at 37°C for 45 minutes with 180 revolution per minute(rpm). To pellet down centrifuged them at 4°C for 10 minutes with a speed 6000g. Removed 700µL of LB and resuspend the pellet in the remaining LB and plated them in Luria agar (LA) plates containing ampicillin (Amp) and incubated at 37°C for 12-16 hours. After the incubation took 100mL of autoclaved LB containing 100µL ampicillin in a conical flask and added one bacterial colony from the LA-Amp plates using a micro tip. Incubated them at 37°C for 12-16 hours with 180 rpm. Upon finishing incubation transferred the bacterial culture into 50mL falcons in the hood and centrifuge them at 4°C at 6000g for 10 minutes. Discard the supernatant completely and done the midi prep using QIAGEN Midi prep kit to extract the plasmid.

Schneider 2 Cell (S2 cell) Maintenance

Schneider 2 cells are one of the common insect cell lines used to express protein *in-vitro*. It is derived from primary culture of late *Drosophila melanogaster* embryo. Took out new batch of S2 cells from cryopreserved tank. Thaw them into room temperature (RT) and them into a 15mL falcon containing 5mL serum containing media (SCM). Centrifuge it at 18°C for 10 minutes with 800 rpm. Discarded most of the supernatant and resuspended the pellet in the 1mL of SCM and transferred into a new flask containing 4mL SCM. Look them under microscope to ensure they are healthy and incubate in the incubator at 24°C. When the cells confluency reaches more than 80% split the cells into two new flasks (add 3mL of SCM and 2mL of old cell culture in each new flasks).

Insect Cell Transfection

Split the cells 24 hours prior to the transfection. After 24 hours transfer them into 12 well plates (500µL each). Thaw serum free media (SFM) and Mirus TransiT2020 (transfection reagent) to room temperature. Added 500µL SFM and add 1µL of plasmid (1µg/µL) in a 1.5mL microcentrifuge tube. Vertex the TransiT2020 reagent a few times and added 2.5µL into the microcentrifuge. Mix them properly and incubated at RT for 30 minutes to ensure plasmid-reagent complex is well formed. Ensure that cells are not floating while doing transfection. Remove the SCM from the wells and add the plasmid-reagent complex. Incubated at in the incubator for 2 hours. Added 500µL SCM and 500µM $CuSO_4$ and kept in the incubator for 24 – 72 hours followed by harvesting and analysis (Fig 2.1).

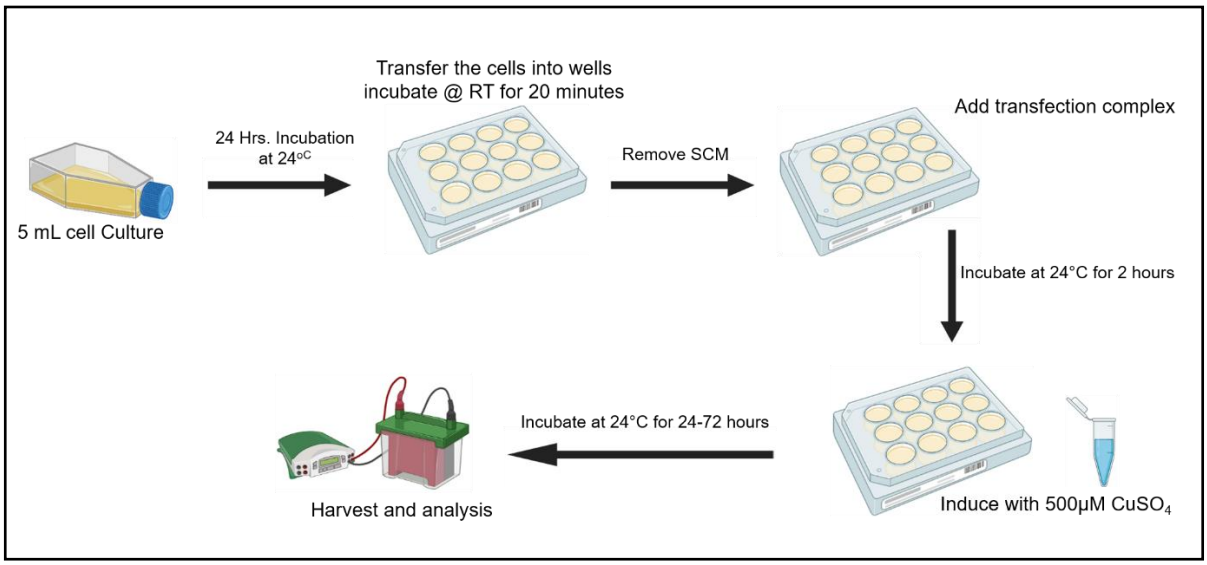


Figure 2.1 Transfection_ *Schematic diagram of transfection in S2 cells*

Lysate Preparation

After the incubation, cells were harvested by centrifugation at 18°C for 10 minutes with 1000 rpm and discard the supernatant. Resuspend the pellets in 100µL of chilled 1x phosphate-buffered saline (PBS). Sonicated that with parameters; 1 second ON, 1 second OFF as impulse, 60% amplitude for 10 seconds. After sonication centrifuge the lysate at 4°C for 1 hour with a speed of 21000 relative centrifugal force (rcf). Separate the supernatant and pellet and resuspend the pellet in 60µL of chilled 1x PBS.

Activity Based Protein Profiling (ABPP)

Prepared lysates were incubated 1µM FP-Rhodamine at 37°C for 45 minutes with 800 rpm in the thermomixer. Reaction mixture was quenched by adding SDS loading dye and incubated at 95°C for 10 minutes with 1000 rpm. Short spin and run them in 10% SDS gel with a mixture of fluorescent and Bio-Rad unstained ladder (1:2). Image the gel using Invitrogen ibright fl1500 imaging system (fluorescent protein).

MS Based Validation of Cloned Plasmids

After doing the ABPP the gel was stained Coomassie brilliant blue stain followed by de-staining and cut of the gel part which have the protein of interest. Chopped the gel into tiny pieces and transferred into an eppendorff containing 500µL of 100% Acetonitrile (ACN) and 500µL of 50mM Ammonium Bicarbonate Buffer (ABC buffer). Kept the samples in a shaker for 24-36 hours at room temperature and periodically changed the buffer (4-5 hours) followed by vacuum filtration. Made 10mM of 5mL Dichlorodiphenyltrichloroethane (DTT) in 50mM ABC buffer and added 500µL into the gel and incubate at 60°C for 30 minutes with 600 rpm. Discard the DDT from the gel and add 100% ACN and incubate at RT for 5 minutes. Remove the supernatant and do the vacuum filtration at 30°C for 5-10 minutes (until the gel become opaque and shrank). Made Iodoacetamide solution (15mg in 4mL ABC buffer) and add 700µL (20mM) into the gels and cover them and incubate at RT for 30 minutes with 600 rpm and discard the supernatant. Done the ACN wash by adding 1mL of 100% ACN and incubate at RT for 5 min then discard the supernatant and vacuum filtered. Remove all the ACN using 200µL micro tip when the gel shrinks and become opaque.

Trypsin Digestion: Took sequencing grade trypsin and added 2mL of filtered ABC buffer followed by vertexing for 40-45 seconds. Added 200µL of trypsin to the gel and incubated at 37°C for 16 hours. Collect the supernatant in a new 1.5mL microcentrifuge tube. Made 2mL of solution containing 10% ACN and 0.4% formic acid in milli-Q water and added to the gels till the gel pieces are sank. Keep them in the thermomixer for 10 minutes at 25°C with 1000 rpm. Made 40% ACN and 0.4% formic acid in milli-Q water and added to the gel pieces and incubated at 25°C for 10 minutes with 1000 rpm. Collected the supernatant in the same 1.5mL microcentrifuge tube which used for collecting supernatant after adding trypsin to the gels. Repeat the same with 100% ACN and collected the supernatant in the same 1.5mL microcentrifuge

tube. Split them between different 1.5mL microcentrifuge tube containing 350-400µL and do the vacuum filtration until it gets completely dry (Store them at -30°C). Add 100µL of 0.1% Trifluoroacetic acid (TFA) to one of the microcentrifuge tubes and mix it properly by pipetting for 1 minutes followed by water bath sonication for 2 minutes. Transfer them to the next tube and repeat the same. After water sonicating the last tube spin the microcentrifuge tube in a table top centrifuge for 2 minutes with maximum speed. Add C18 piece into a new 200µL micro tip and gently press it down. To activate add 50µL of 100% ACN and press down using syringe. Add 50µL of 0.1% TFA to equilibrate and press down gently with a syringe. Add the samples to the C18 column, remove the bubbles, and let the sample pass through. Add 100µL of 0.1% TFA. Take a new autoclaved 1.5mL microcentrifuge tube and put the column in it and add 50µL of 60% ACN + 0.1% TFA solution and elute (Don' press down). After the elution do the vacuum centrifugation at 30°C until it becomes completely dried.

Competitive ABPP

The CG15111 lysates were treated with 20µM Tetrahydrobiopterin (THL), CG1309 lysates were treated with 20µM KC01 and controls were treated with dimethyl sulfoxide (DMSO) and incubated at thermomixer at 37°C for 2 hours. Then all samples were treated with 1µM FP-Rhodamine and incubated at 37°C for 1 hour with 800 rpm. Followed by denaturing using SDS loading dye (12.5µL of 5x SDS loading dye for 30µL lysate) and incubated at 95°C for 12.5 minutes with 1000 rpm. Short spin and run them in 10% SDS gel with a mixture of fluorescent and Bio-Rad unstained ladder (1:2). Image the gel using Invitrogen ibright fl1500 imaging system (fluorescent protein).

Bacterial Clearance Test (BCT)

5 days old flies are used for this experiment. Flies were infected with *Staphylococcus saprophyticus* bacteria which were overnight cultured (OD = 0.5) with novobiocin antibiotic. We have done this experiment with two different time points one is 0^{th} hour (immediately after infection) and the other is 6^{th} hour (after 6 hours of infection). After infection immediately transfer the flies to a 1.5mL microcentrifuge containing 70% ethanol and within 5 minutes wash them with distilled water and paint brush. Use soft paper towel to dry them. Transfer 4 flies into a new 1.5mL microcentrifuge containing 100µL LB and crush them well using pestles. Plate them in Novobiocin-LA plates and

incubate at 37°C for 14 hours and count the number of colonies. Repeated the same for 6th hour flies post 6 hours of infection (Fig 3.2).

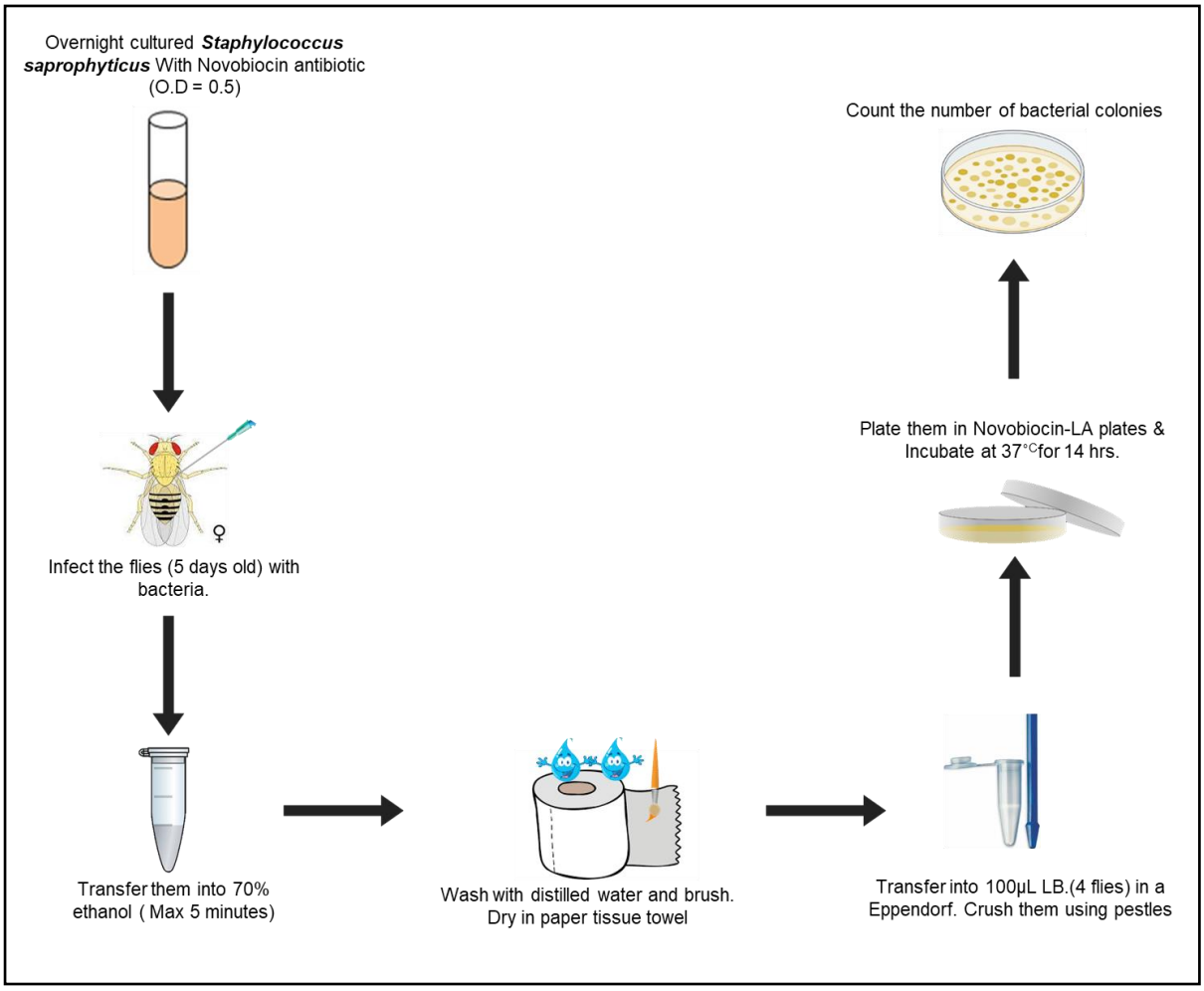


Figure 2.2 Bacterial Clearence Test_ *Schematic diagram of BCT Protocol*

3. RESULTS

3.1 Phospholipases in *Drosophila melanogaster*

3.1.1 Classification of Phospholipase in *Drosophila melanogaster*

Classified phospholipases found in *Drosophila melanogaster* based on their site of cleavage using fly base data.

PHOSPHOLIPASE A1				
CG17191	CG6271	sxe2	CG7367	CG34447
CG17192	CG6277	CG13282	CG6675	CG34448
CG6295	CG6296	CG10163	CG4582	CG6472
CG6283	PAPLA1	CG4267	CG10357	
PHOSPHOLIPASE A2				
CG11029	CG42237	iPLA2- VIA	CG18858	
CG3009	CG14507	PGAP1	CG31683	
PHOSPHOLIPASE B				
CG15111	CG7365	CG11029	Apt1	
PHOSPHOLIPASE C				
Plc21c		sl		
PHOSPHOLIPASE D		**PHOSPHOLIPASE ACTIVITY**		
dob		CG1309		

Orthogonal gene has phospholipase

Phosphatidylinositol phospholipase A2

Phosphoinositide phospholipase C

Table 3.1 Phospholipase in Drosophila_ *Classification of phospholipases found in Drosophila.*

3.1.2 Phylogenetic tree of Phospholipase in *Drosophila melanogaster*

Using the fasta data collected from National center for biotechnology information (NCBI) generated a phylogenetic tree with the help of megaX software (Fig 3.1)

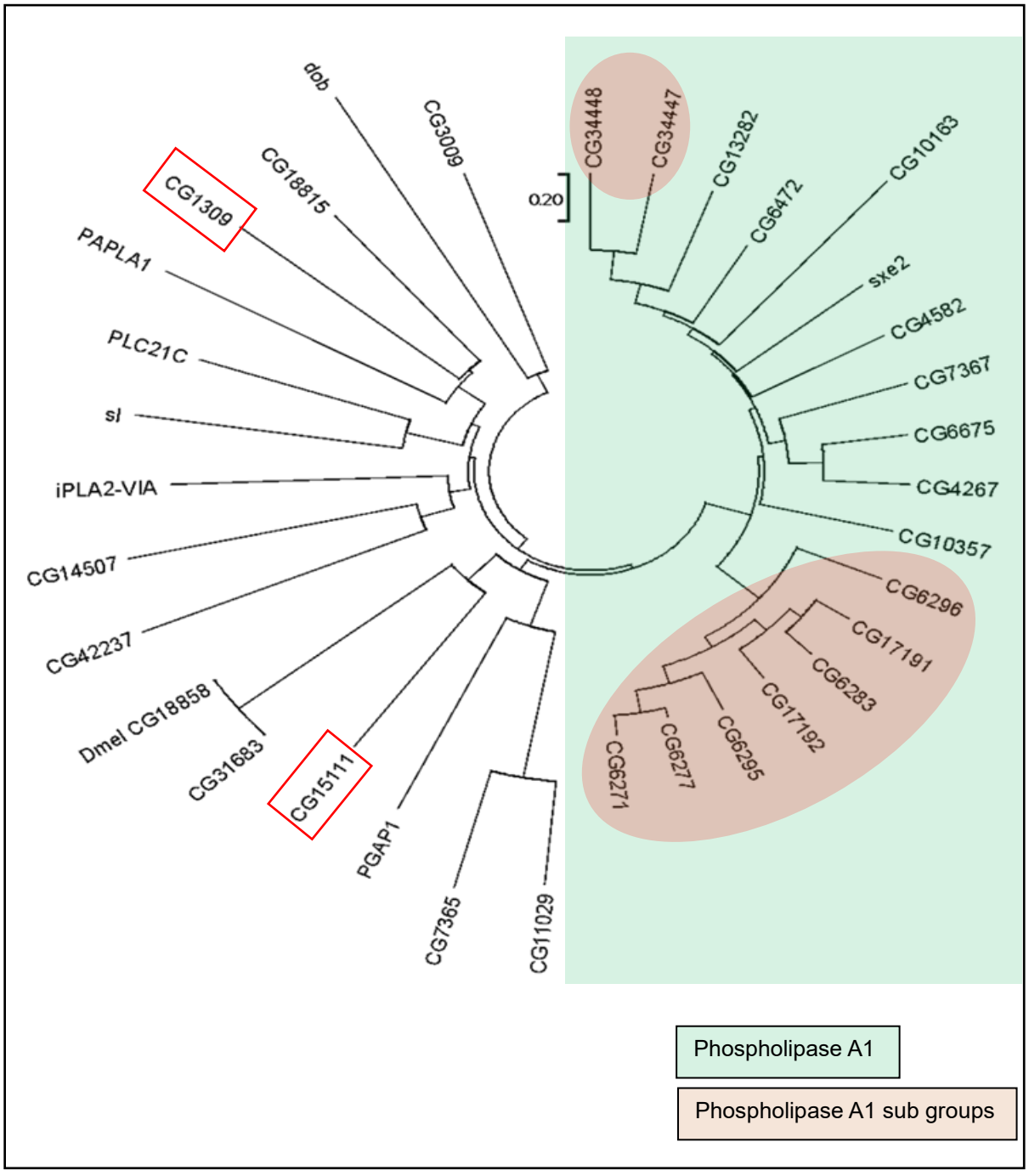


Figure 3.1 Phylogenetic tree_ *Phylogenetic tree of Phospholipases found in Drosophila melanogaster*

3.1.3 Cytogenetic Map of Phospholipases in *Drosophila melanogaster*

Tabulated the cytogenetic position of different phospholipases found in *Drosophila melanogaster* with the help of fly base data.

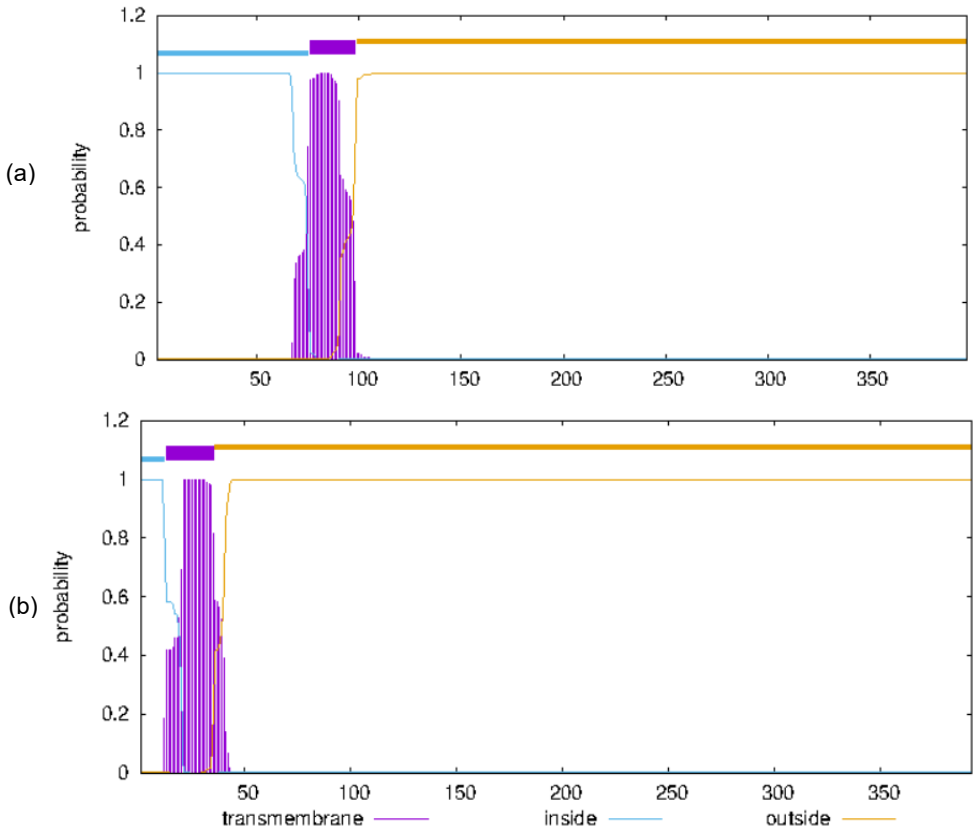
sxe2	3R:16,083,878..16,087,222 [+]	Plc21C	2L:305,935..355,566 [+]	Apt1	3L:11,628,037..11,630,749 [+]
CG4582	3R:25,581,223..25,583,179 [-]	CG4267	2L:2,242,378..2,245,274 [+]	CG7365	3L:20,124,298..20,127,381 [-]
CG6296	3R:27,003,236..27,005,469 [-]	CG34448	2L:2,416,792..2,417,965 [-]	CG10357	3L:3,615,382..3,616,682 [+]
CG6295	3R:27,005,899..27,007,055 [-]	CG34447	2L:2,418,422..2,420,013 [-]	CG1309	3L:4,259,430..4,261,643 [+]
CG17192	3R:27,017,180..27,018,287 [-]	CG11029	2L:5,746,157..5,747,961 [+]	CG10163	3L:6,257,423..6,258,760 [-]
CG17191	3R:27,018,608..27,019,751 [-]	CG7367	2L:8,026,914..8,039,788 [-]	iPLA2-VIA	3L:9,860,217..9,864,349 [-]
CG6283	3R:27,020,238..27,021,378 [-]	PAPLA1	2L:8,134,247..8,159,638 [-]	CG3009	X:4,658,158..4,666,700 [-]
CG6277	3R:27,022,071..27,023,217 [-]	CG13282	2L:16,832,065..16,834,502 [-]	PGAP1	X:5,685,253..5,689,473 [-]
CG6271	3R:27,024,968..27,026,109 [-]	CG31683	2L:20,447,734..20,449,964 [-]	CG42237	X:13,115,131..13,123,066 [+]
CG14507	3R:29,226,204..29,227,725 [-]	CG18858	2L:20,456,847..20,459,075 [-]	dob	X:15,067,007..15,069,091 [+]
CG15111	2R:19,137,372..19,141,034 [+]	CG6675	2L:22,108,964..22,110,449 [+]	sl	X:16,350,315..16,356,482 [+]
CG6472	2R:16,880,713..16,884,695 [+]				

Table 3.2 Cytogenetic map_ *Cytogenetic map of Phospholipase in Drosophila.*

3.2 Fly Genes and Mammalian Orthologs

3.2.1 Transmembrane Domain Similarity with Mammalian orthologs

Using TMHMM - 2.0 found the predicted transmembrane domains of fly and their mammalian orthologs (Fig 3.2 a,b,c,d).



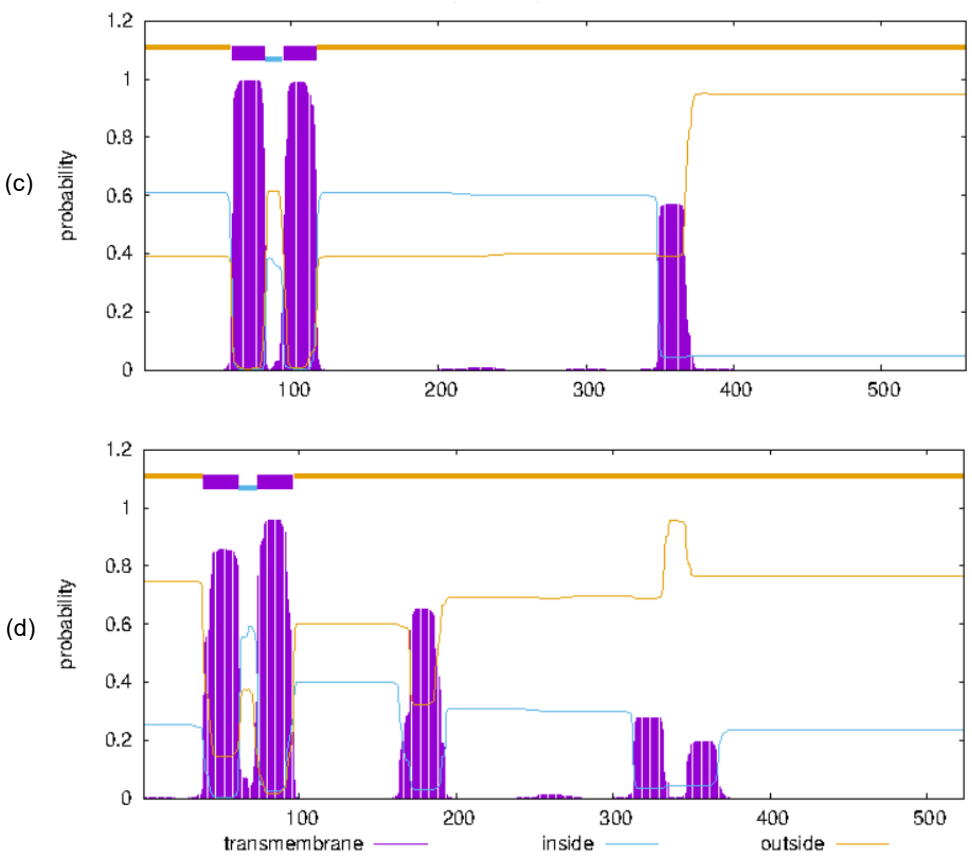


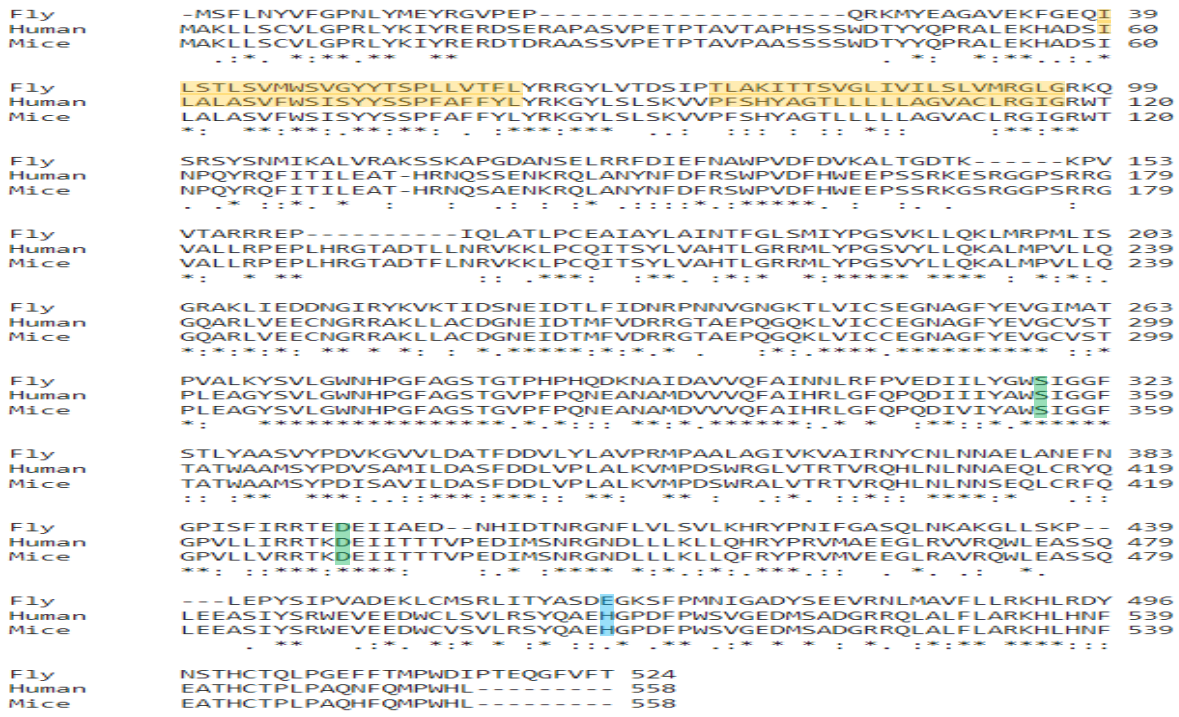
Figure 3.2 Transmembrane domains_ *(a) Human ABHD12, (b) CG15111, (c) Human ABHD16A, (d) CG1309*

3.2.2 Sequence Similarity with Mammalian Orthologs

Using clustal omega found the sequence similarity of CG15111 & CG1309 with their mammalian orthologs human and mice, (Fig 3.3 a,b).

```
Fly       ------------------------------------------------------------MLFTRR  6
Human     MRKRTEPVALEHERCAAAGSSSSGSAAAALDADCRLKQNLRLTGPAAAEPRCAADAGMKR  60
Mice      MRKRTEPVTLEHERCAASGSSSSGSAAAALDADCSLKQNLRLAGKGTAEPHSASDAGMKR  60
                                                                     :*

Fly       RLLKRVGLR-CLQACLLIFFLIFVVLPLIFRYSVTFQRGILFLTFIKYPKGLDLTKPESV  65
Human     ALGRRKGVWLRLRKILFCVLGLYIAIPFLIKLCPGIQAKLIFLNFVRVPYFIDLKKPQDQ  120
Mice      ALGRRKSLWFRLRKILLCVLGFYIAIPFLVKLCPGIQAKLIFLNFVRVPYFIDLKKPQDQ  120
           * :*  .:    *:  *: .: ::::.*::.:  .  :*  ::**.*:: *  :**.**:.

Fly       GLYATRNFYITVKDHDQDEDGVRVGVWHVLPSNAVRRFKRELRVEEEVAQDPDQQLDPAP  125
Human     GLNHTCNYYLQ------PEEDVTIGVWHTVPAVWWKNAQ---------------------  153
Mice      GLNHTCNYYLQ------PEDDVTIGVWHTIPSVWWKNAQ---------------------  153
          **   * *:*:         *:.* :****.:*:   :. :

Fly       GNERELKELSPAIRSEFPVVLPENEQLFYERLLRMPGGTVVLYLHGNTASRGSGHRSEVY  185
Human     ----------------------GKDQMWYEDALA-SSHPIILYLHGNAGTRGGDHRVELY  190
Mice      ----------------------GKDQMWYEDALA-SNHAIILYLHGNAGTRGGDHRVELY  190
                                 ::*::**   *   .  ::******:.:**..** *:*

Fly       KLLRKLNYHVFSFDYRGYADSDPVPPTEEGVVRDAMMVFEYIAN-TTSNPIVVWGHSLGT  244
Human     KVLSSLGYHVVTFDYRGWGDSVGT-PSERGMTYDALHVFDWIKARSGDNPVYIWGHSLGT  249
Mice      KVLSSLGYHVVTFDYRGWGDSVGT-PSERGMTYDALHVFDWIKARSGDNPVYIWGHSLGT  249
          *:* .*.***.:*****:.**   . *:*.*:. **: **::*    : .**: :*******

Fly       GVATHLCAKLASLRERAPRGVILESPFTNIRDEIRMHPFAKLYKNLPWFNFTISQPMYTN  304
Human     GVATNLVRRLC-ERETPPDALILESPFTNIREEAKSHPFSVIYRYFPGFDWFFLDPITSS  308
Mice      GVATNLVRRLC-ERETPPDALILESPFTNIREEAKSHPFSVIYRYFPGFDWFFLDPITSS  308
          ****:*  :*.  **  * .:**********:* : ***: :*: :* *:: : :*: :.

Fly       RLRFESDVHVLEFRQPIMIIHAEDDVVVPFNLGYRLYRIALDGRSRTSGPVEFHRFGASR  364
Human     GIKFANDENVKHISCPLLILHAEDDPVVPFQLGRKLYSIAAPARSFRDFKVQFVPFHSDL  368
Mice      GIKFANDENMKHISCPLLILHAEDDPVVPFHLGRKLYNIAAPSRSFRDFKVQFIPFHSDL  368
           ::* .* :: .:  *::*:***** ****:** :** **  .**  .  *:*  * :.

Fly       KYGHKYLCRAPELPGLIQKFVENYRDAVY-       393
Human     GYRHKYIYKSPELPRILREFLGKSEPEHQH       398
Mice      GYRHKYIYKSPELPRILREFLGKSEPERQH       398
```

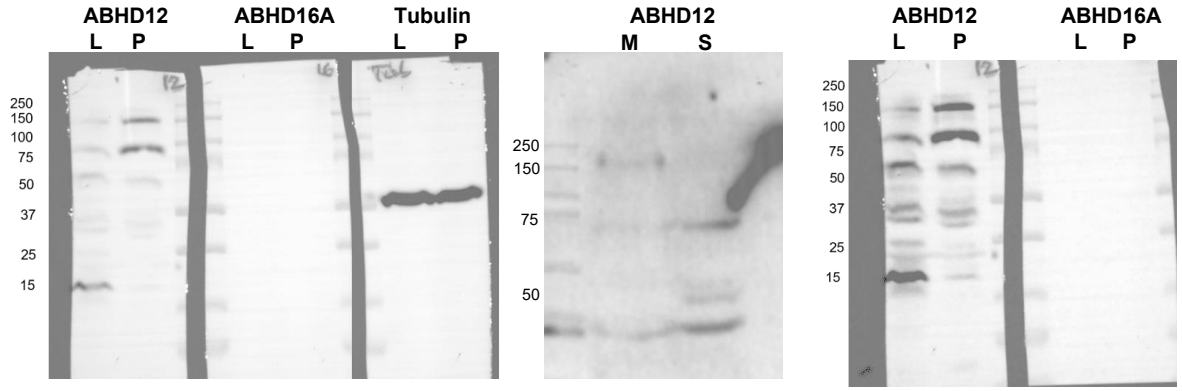
(a)



(b)

***Figure 3.3* Multiple sequence alignment_** *(a) Multiple sequence alignment CG15111 with mammalian ABHD12 with transmembrane domain and conserved catalytic center, (b) Multiple sequence alignment CG1309 with mammalian ABHD16A with transmembrane domain and conserved catalytic center*

3.3 Affinity of Mammalian Antibody towards Fly Protein



Transfer Condition	4°C, 90v, 90 Minutes
Primary antibody	Anti ABHD12 and Anti ABHD16A
Secondary antibody	αRb - HRP

S	Soluble fraction
M	Membrane fraction

Tubulin	Positive control
P	Pupa
L	Larva

Figure 3.4 Affinity of mammalian antibodies towards fly proteins_ *Western blot image for analysing the affinity of mammalian antibodies towards fly proteins.*

3.4 Generation of Recombinant Plasmid Using SLiCE Cloning

3.4.1 Addition of N' 6x His tag and PCR Based Mutagenesis

All genes with N' 6x His tag and pRM vector are amplified using PCR. All gene ends were made homologous to pRM vector using PCR with primers mentioned in earlier (Table 2.2). Serine to Alanine mutants were made using PCR based mutagenesis with mutagenesis primers. All PCR products were confirmed through agarose gel electrophoresis using 0.8% agarose gel made in 1% Tris-acetate-EDTA (TAE) buffer with HI media 100 base pair(bp) ladder for genes of interest and 1kilo base pair ladder (kb) for pRM vector (Fig 3.5).

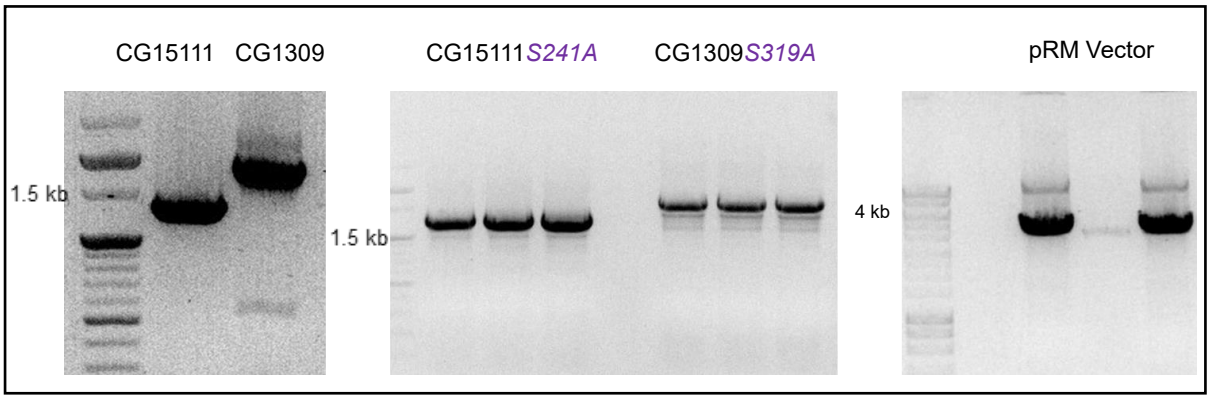


Figure 3.5 ***PCR amplification***_*gene of interest with N' 6x His tag and pRM vector.*

3.4.2 SLiCE Cloning

All genes were successfully cloned to S2 cells expressing pRM vector with a N' terminal 6x His tag. Cloning was confirmed through colony PCR followed by 0.8% agarose gel electrophoresis with 100bp DNA ladder (Fig 3.6) and Sanger sequencing with appropriate primers (Fig 3.7)

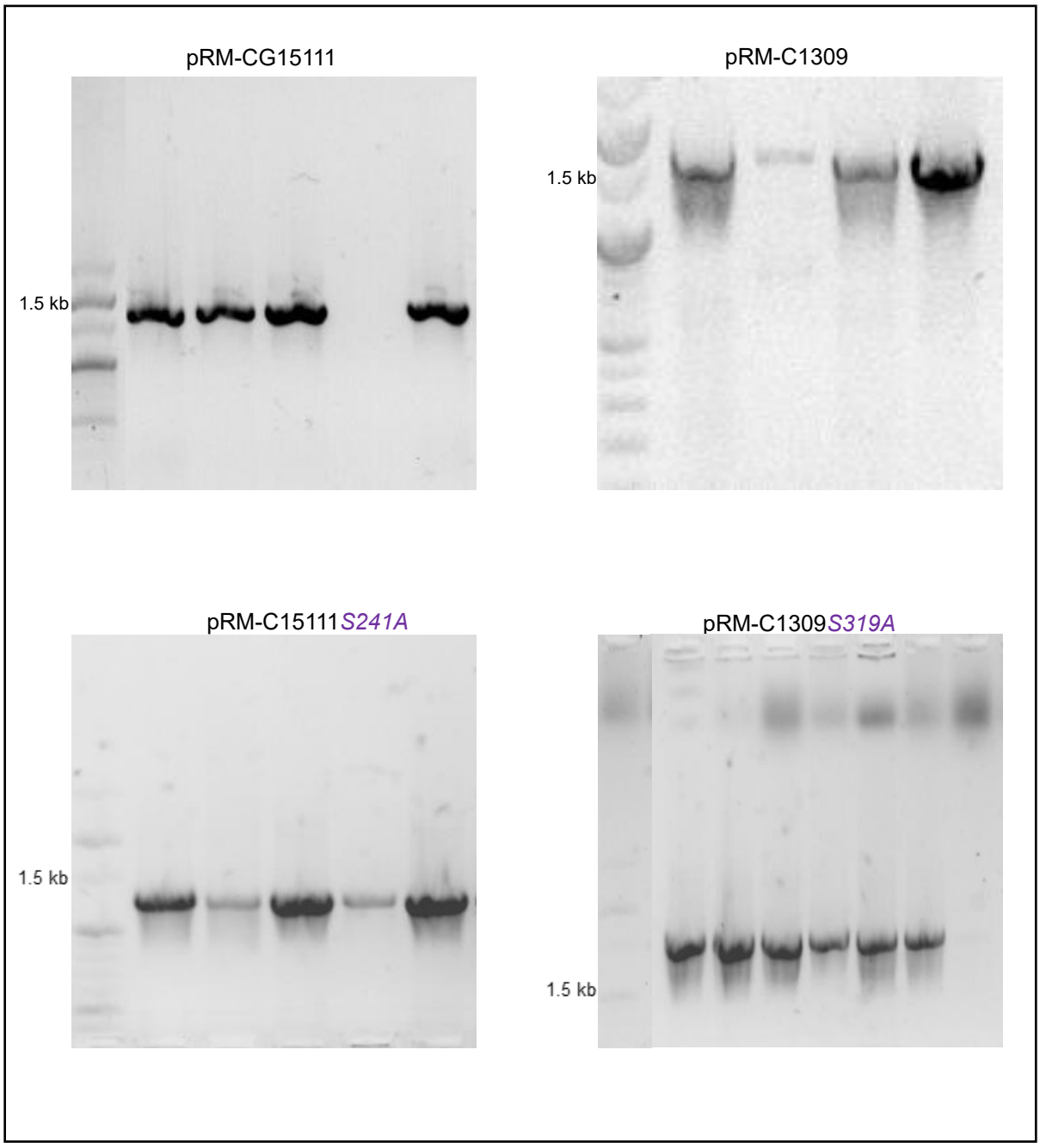


Figure 3.6 Colony PCR_ *PCR products in 0.8% agarose gel electrophoresis*

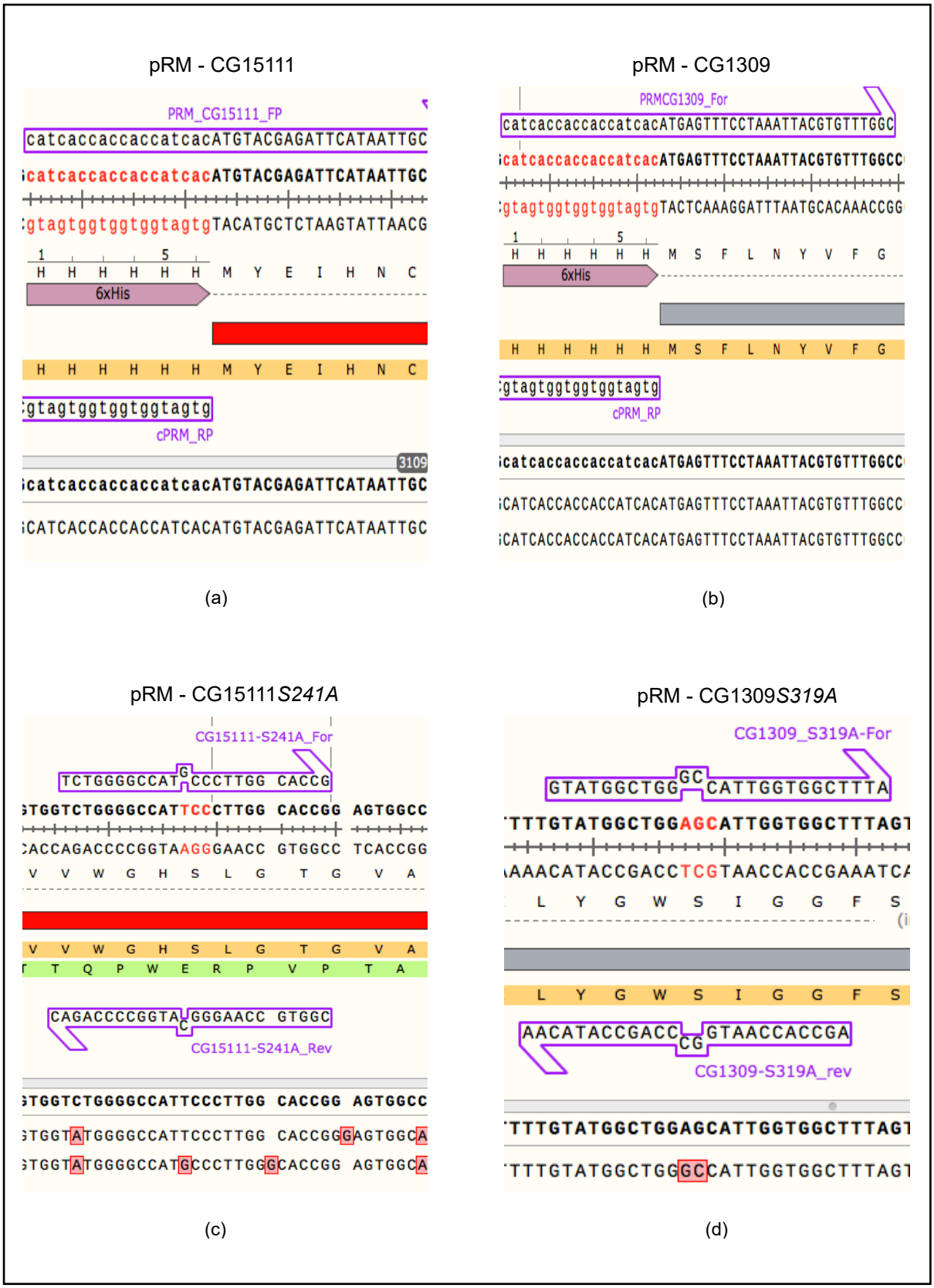


Figure 3.7 Sanger sequencing results of cloned plasmids_ (a) *pRM-CG15111* ***(b)*** *pRM-CG1309* ***(c)*** *pRM-CG1511S241A* ***(d)*** *pRM-CG1309S319A*

All the plasmids are further transformed into DH5α (E. coli strain) followed by 100mL primary culture with ampicillin antibiotic (12-16 hours incubation) for obtaining higher concentration. DH5α without any plasmid insertion is used as a control. All bacterial cultures are purified using QIAGEN Plasmid Plus Midi prep kit (used the standard protocol provided by QIAGEN) and quantified using Nanodrop (Table 3.3). Purified samples were run in a 0.8% agarose gel at 110V to confirm the successful transformation.

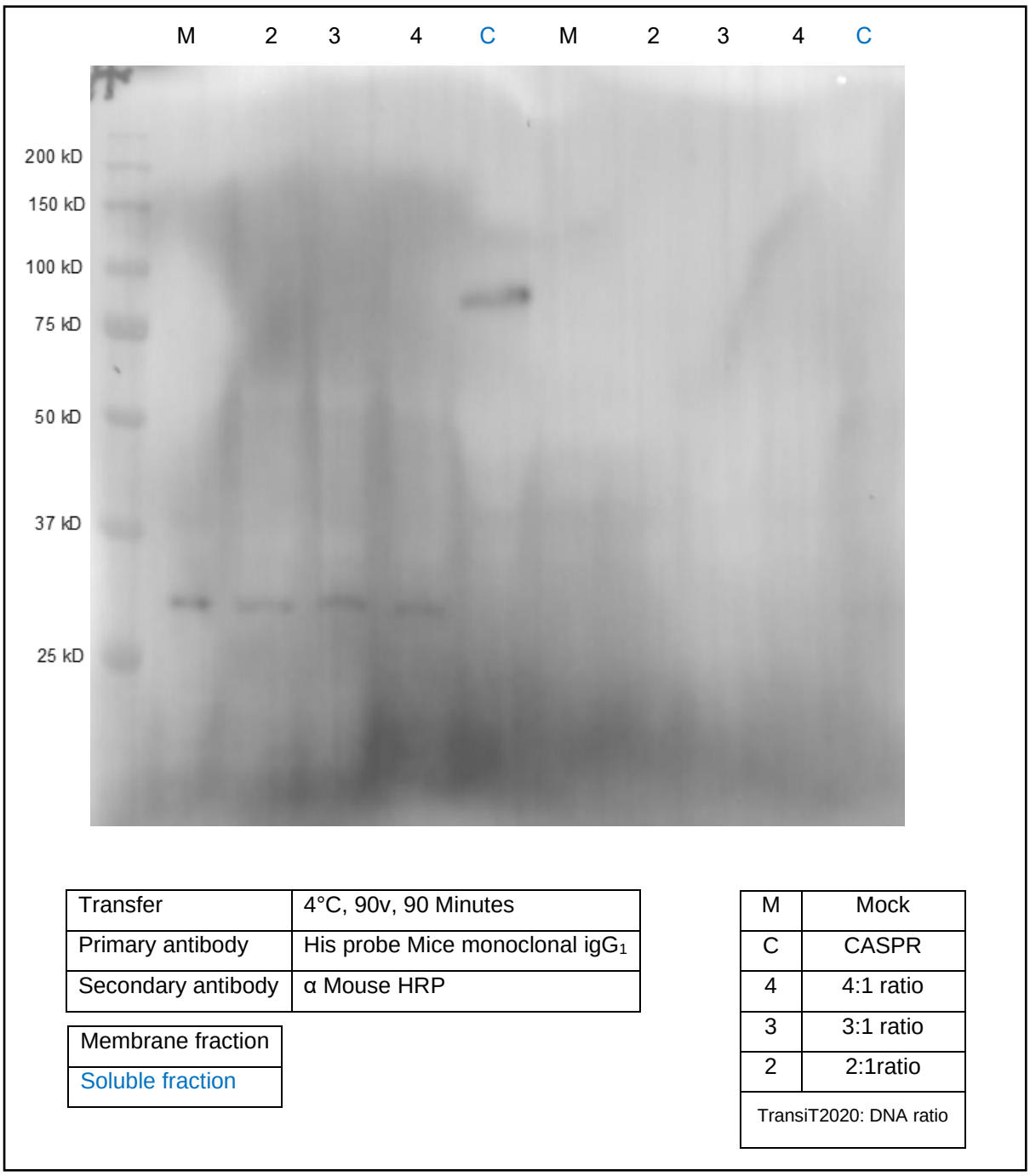
Plasmid	Concentration (ng/μL)	Total Yield (μg)
CG15111 (1)	2361.1	472.220
CG15111 (2)	2216.8	443.360
CG1309 (1)	3739.1	747.820
CG1309 (2)	3006.6	601.320
CG15111*S241A* (1)	2509	501.800
CG15111*S241A* (2)	2127.1	425.420
CG1309*S319A* (1)	1145.9	229.180
CG1309*S319A* (2)	1226.9	245.380

Table 3.3 Plasmid Midiprep_ *quantification of transformed plasmids clones*

3.5 Insect Tissue Culture

3.5.1 Standardization of In Vitro Expression Using CG15111

Western blot image of CG15111 with varying TransiT2020: DNA ratio and CASPR as positive control (Fig 3.8). DNA concentration is fixed 1µg/µL and ladder is Bio-Rad pre-stained protein ladder.



Transfer	4°C, 90v, 90 Minutes
Primary antibody	His probe Mice monoclonal igG_1
Secondary antibody	α Mouse HRP

Membrane fraction
Soluble fraction

M	Mock
C	CASPR
4	4:1 ratio
3	3:1 ratio
2	2:1ratio
TransiT2020: DNA ratio	

Figure 3.8 Standardization of in vitro expression_ *In vitro expression in S2 cells using CG15111with varying volume ratios of TransiT2020 to DNA.*

3.5.2 Standardization of In Vitro Expression Using Cloned Plasmids

Tried to standardize the in vitro expression using all the cloned plasmids with CASPR as a positive control. TransiT2020: DNA ratio is 2:1 and ladder used was Bio-Rad pre stained protein ladder (Fig 3.9).

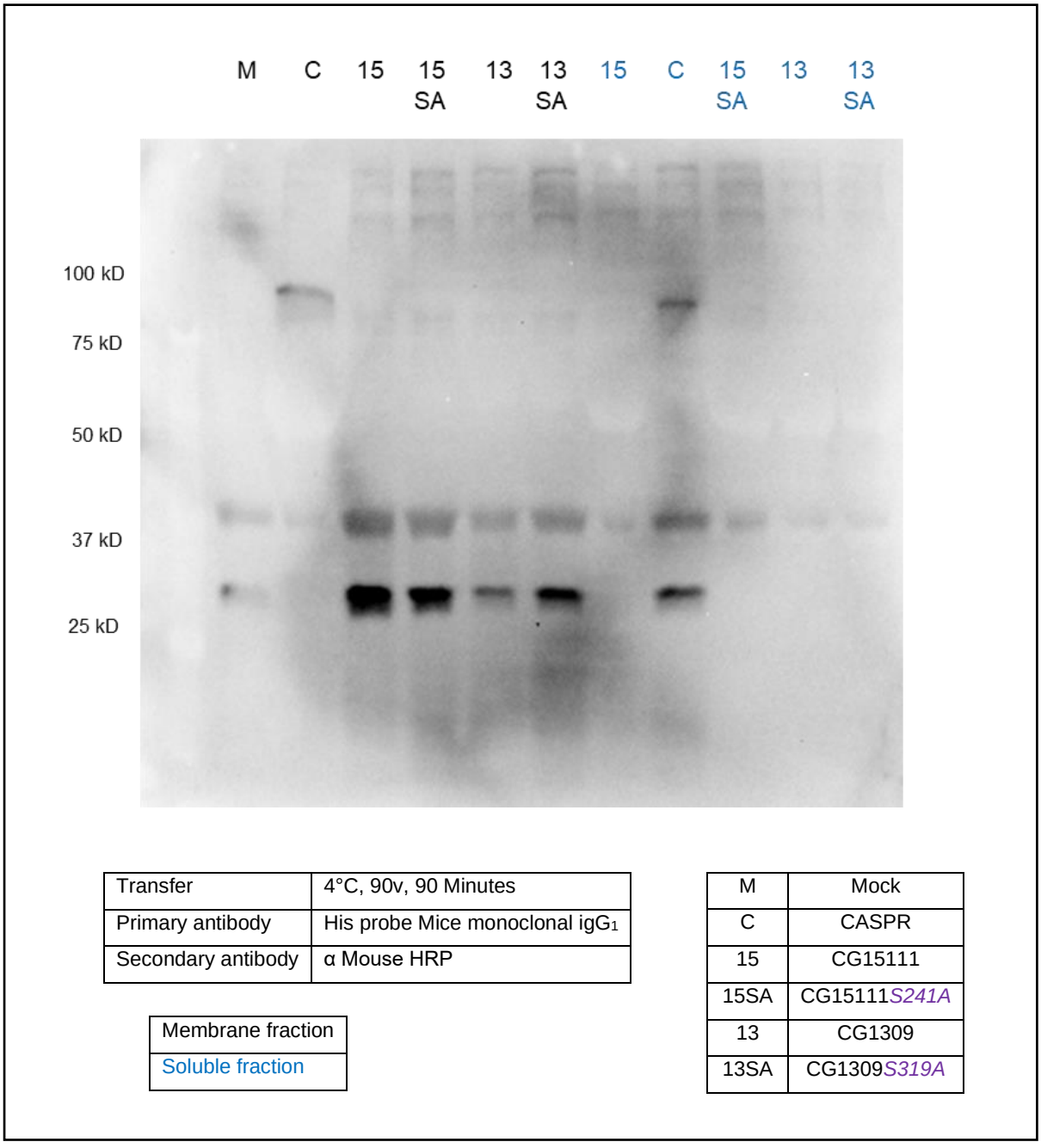


Figure 3.9 Standardization of in vitro expression_ *in S2 cells using all cloned plasmids and CASPR as positive control*

3.5.3 Checking the Expression Activity Based Protein Profiling (ABPP)

The plasmids were expressed transiently and analysed the product using western blot (Fig 3.10a) and ABPP (Fig 3.10b). The transfection was repeated multiple times and ABPP analysis was reproduced (Fig 3.11) with a mixture of fluorescent and Bio-Rad unstained ladder (1:2).

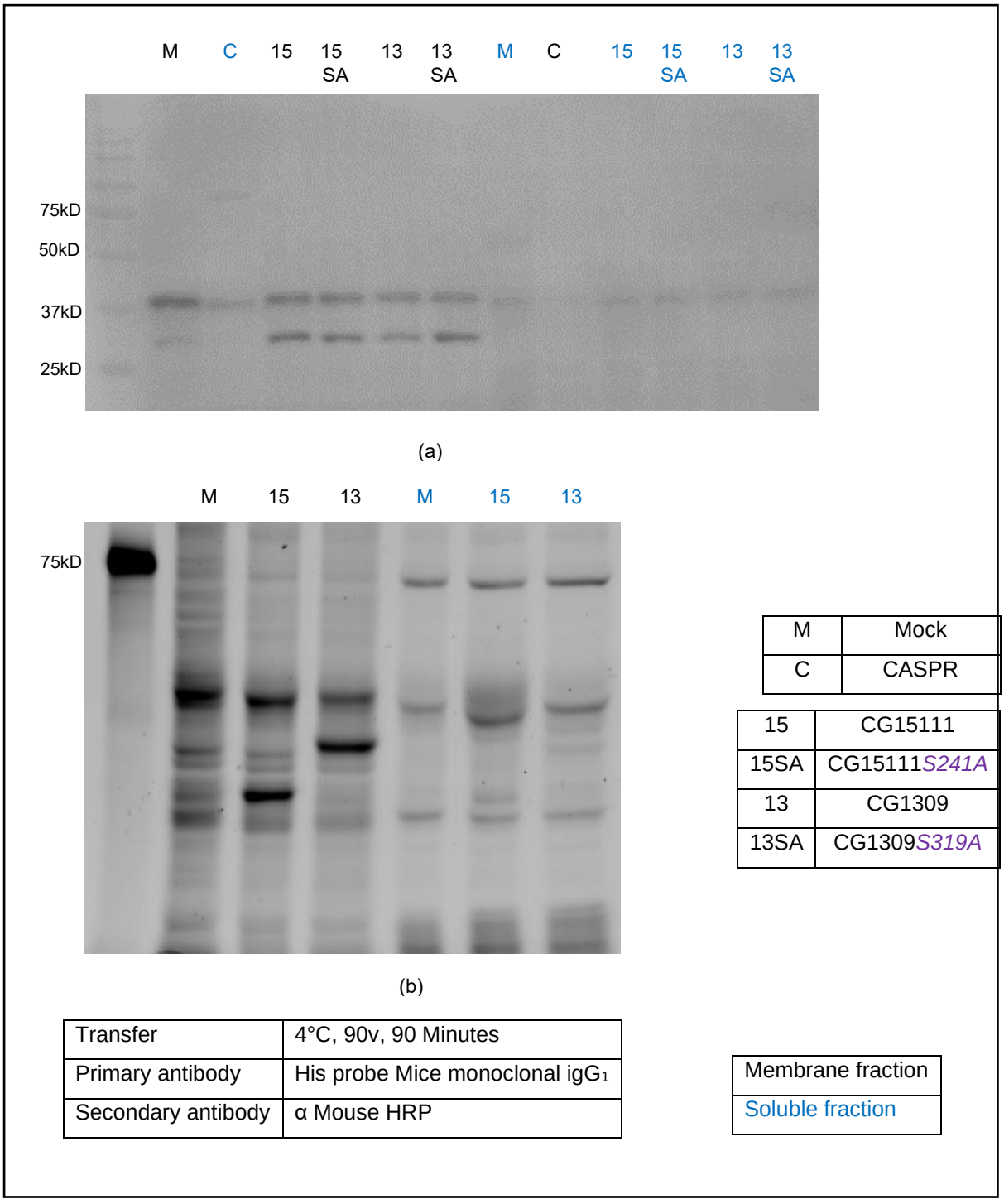


Figure 3.10 Standardisation of In vitro expression *a) Western blot image of standardization of in vitro expression of cloned plasmids.*
(b) ABPP analysis for checking serine hydrolase activity in cloned plasmid expression.

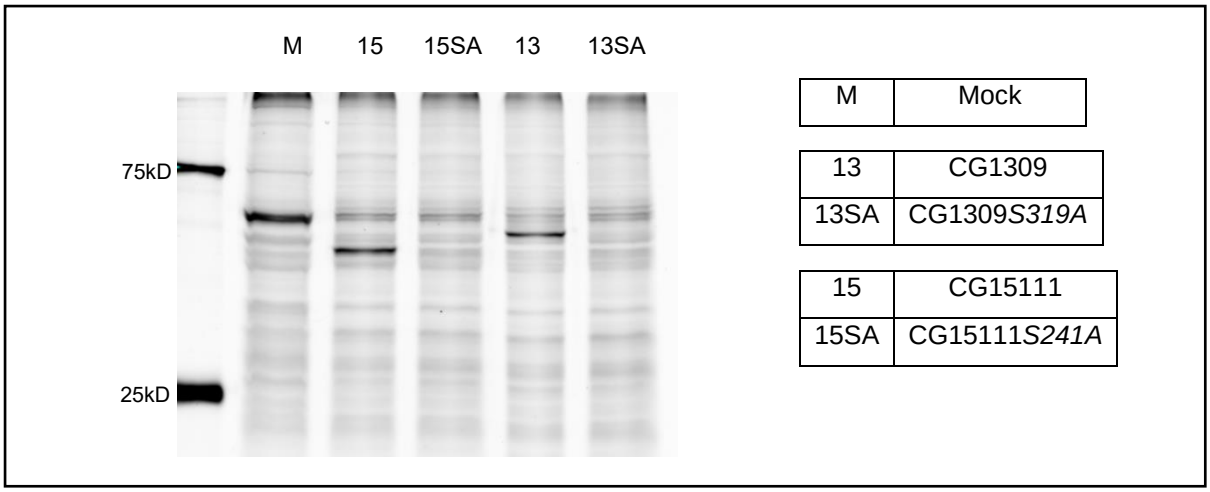


Figure 3.11 ABPP analysis_ *of invitro expressed cloned plasmids.*

3.5.4 Standardizing the Incubation Time for ABPP Analysis

Transfection was carried out with the cloned plasmids with varying incubation time of 24, 48, 72 hours with maintaining all other conditions same with CG15111 (Fig 3.12).

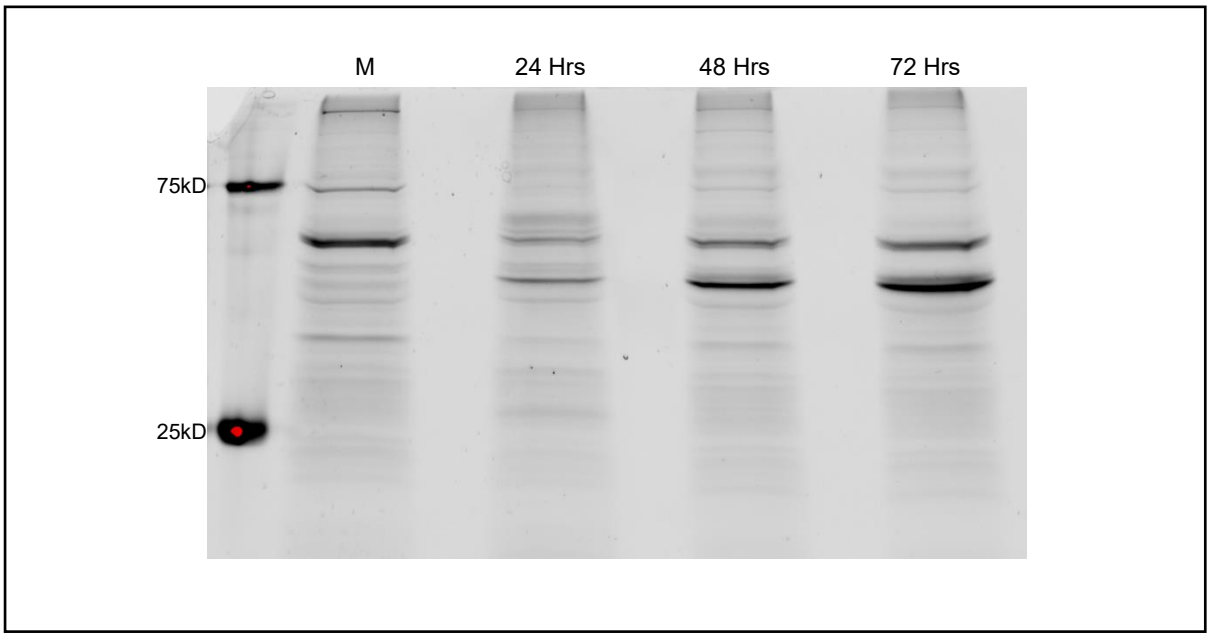
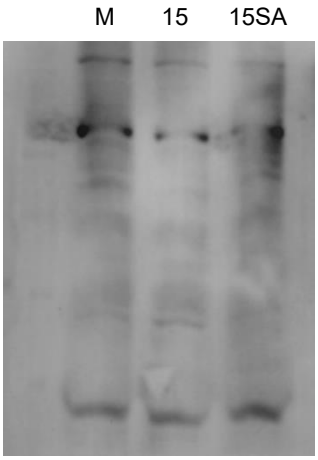


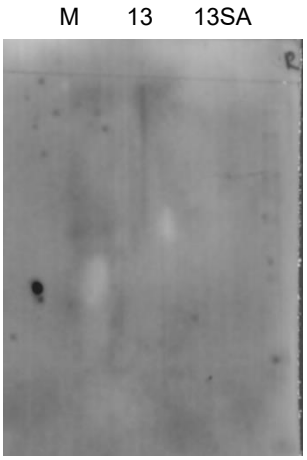
Figure 3.12 ABPP analysis_*gel image of CG15111 in vitro expression with different incubation time.*

3.5.5 Affinity of Mammalian Antibodies towards In Vitro Expressed Proteins

Mammalian antibodies doesn't have any affinity towards *in vitro* expressed CG15111 and CG1309 (Fig 3.13).



(a)



(b)

Transfer	4°C, 90v, 90 Minutes
Primary antibody	Anti ABHD12, Anti ABHD16A
Secondary antibody	α Rabbit HRP

M	Mock
15	CG15111
15SA	CG15111*S241A*
13	CG1309
13SA	CG1309*S319A*

Figure 3.13 Western blot image of in vitro expressed protein_ *(a) in vitro expressed CG15111 with ABHD12 specific antibody. (b) in vitro expressed CG1309 with ABHD16A antibody*

3.6 C Terminal 6x His Tag and Expresing In Vitro in S2 Cells

3.6.1 Generation of C terminal 6x His tag Plasmid

Generated C terminal 6x His tag construct for CG1309 and transformed into DH5α followed by primary culture and mini prep purification. The yield is 2.54µg and successful cloning confirmed through colony PCR (Fig 3.14) and Sanger sequencing (Fig 3.15).

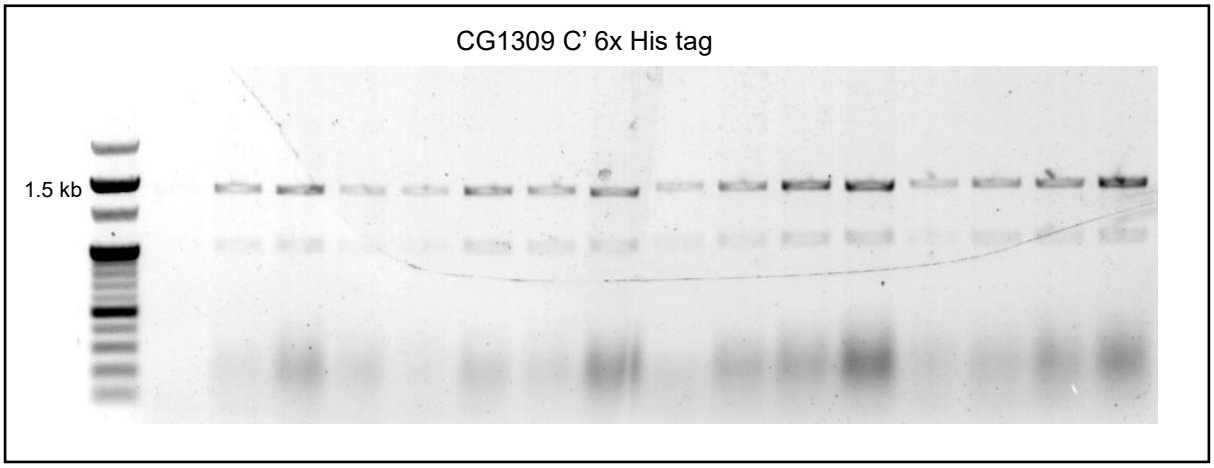
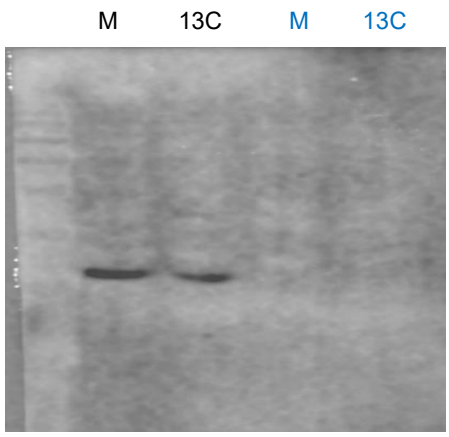


***Figure 3.15 Agarose gel electrophoresis**_gel image of C terminal 6x His tag pRM-CG1309 plasmid colony PCR*

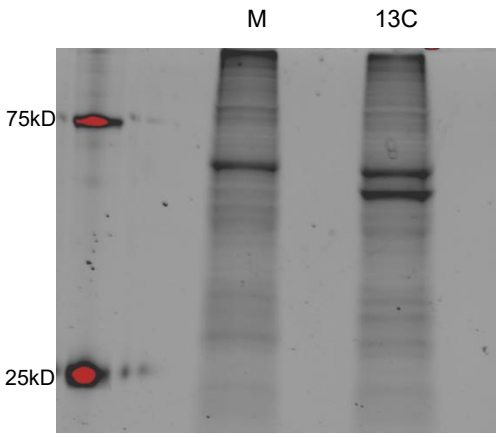
***Figure 3.14 Sanger sequencing**_results pRM-CG1309 C' 6x His tag plasmid*

3.6.2 In Vitro Expression of C' 6x His Tag pRM-CG1309 in S2 Cells

C terminal 6x His tagged pRM CG1309 shows no band in western blot (Fig 3.16a) and shows positive band in ABPP analysis (Fig 3.16b).



(a)



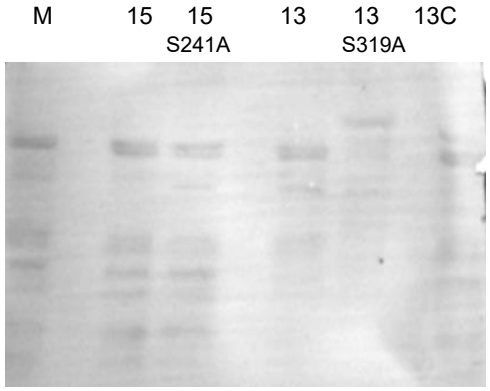
(b)

Transfer	4°C, 90v, 90 Minutes
Primary antibody	His probe Mice monoclonal igG_1
Secondary antibody	α Mouse HRP

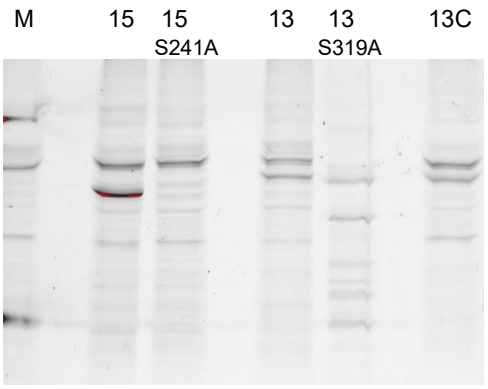
M	Mock
13C	CG1309 C terminal tag

Figure 3.16 C terminal tag expression_*a) Western blot image of in vitro expressed pRM-CG1309 with C' 6x His tag (b) ABPP gel image of in vitro expressed pRM-CG1309 with C' 6x His tag*

Repeated the transfection and protein lysate analyzed using western blot (Fig 3.17a) and ABPP (Fig 3.17b)



(a)

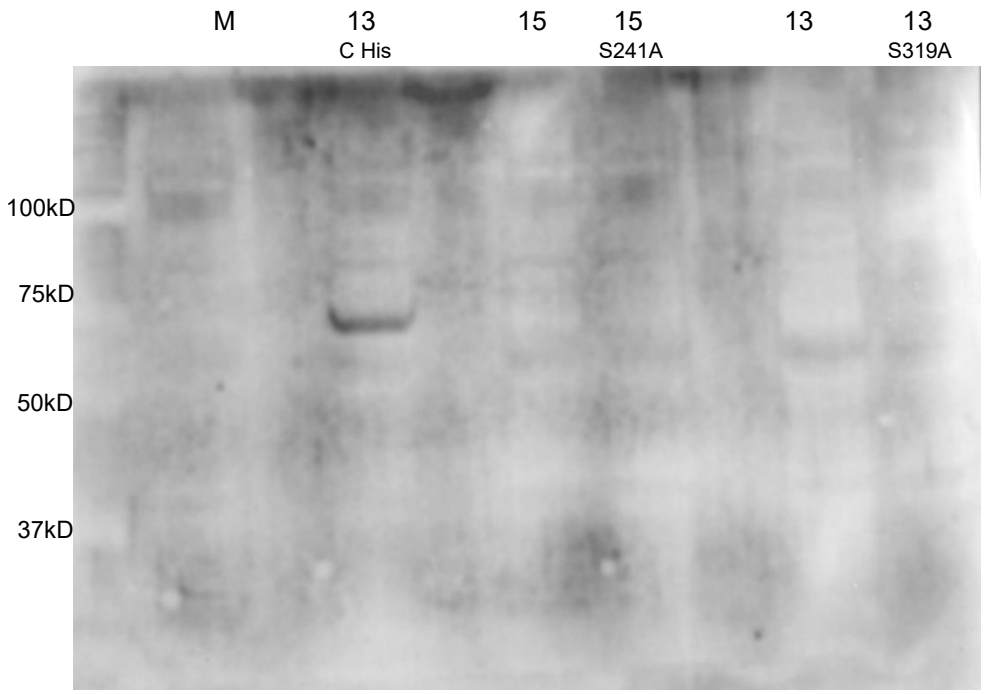


(b)

Transfer	4°C, 80v, 3 hours
Primary antibody	His probe Mice monoclonal igG_1
Secondary antibody	α Mouse HRP

Figure 3.17 In viro expression of C terminal tagged plasmids_ *a) Western blot image of in vitro expressed pRM-CG1309 with C' 6x His tag*
(b) ABPP gel image of in vitro expressed pRM-CG1309 with C' 6x His tag

Replicated the transfection and western blot analysis with different antibodies and observed a positive band for C' 6x His tag plasmid Fig (3.18).



Transfer	4°C, 80v, 3 hours
Primary antibody	His probe rabbit polyclonal igG_1
Secondary antibody	α rabbit HRP

Figure 3.18 Western blot image_*of in vitro expressed pRM-CG1309 with C' 6x His tag*

3.7 C Terminal FLAG Tagged Plasmids and Expression in S2 cells

3.7.1 Addition of FLAG Tag and PCR Amplification

0.8% agarose gel electrophoresis image of all amplified genes with C terminal FLAG tag with ends homologous to pRM vector and amplification of pRM vector (Fig 3.19 a, b).

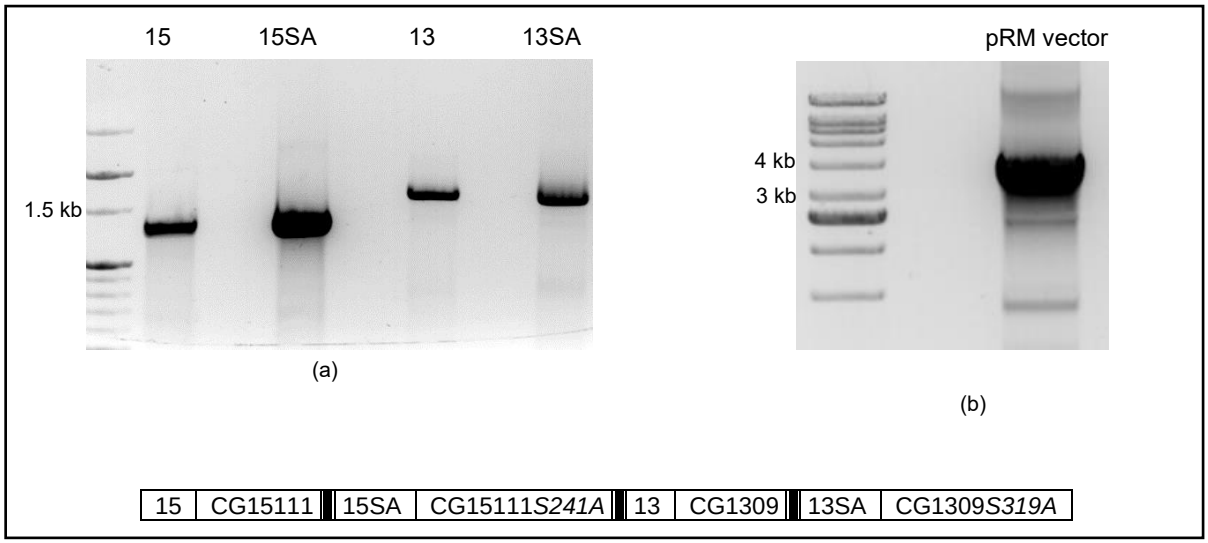


Figure 3.19 Agarose gel electrophoresis_*image of (a) all genes with FLAG tag (b) Amplified pRM vector.*

3.7.2 SLiCE Cloning of FLAG Tagged Genes into pRM Vector

Except CG15111 all genes with C' FLAG tag was cloned into pRM vector which was confirmed through colony PCR (Fig 3.20) and Sanger sequencing (Fig 3.21).

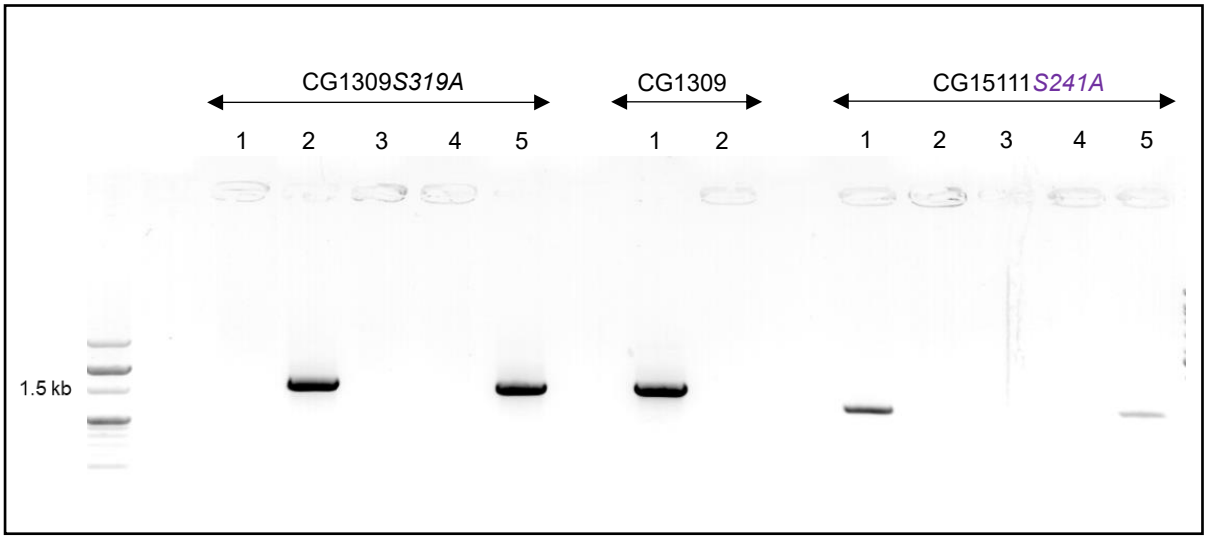


Figure 3.20 Colony PCR_ *products in 0.8% agarose gel electrophoresis*

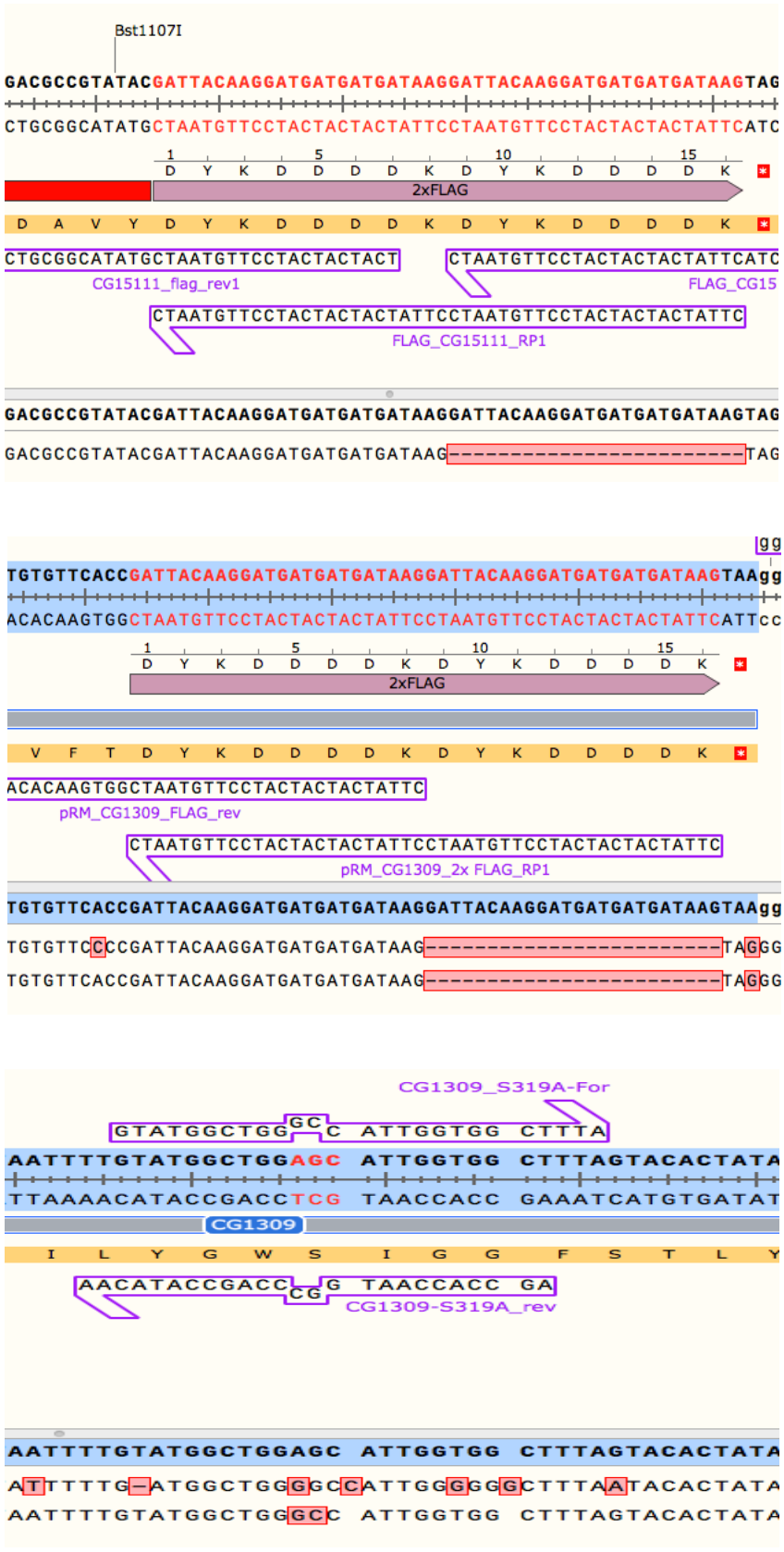


Figure 3.21 Sanger sequencing_*result of FLAG tagged plasmids (a) CG15111S241A, (b) CG1309, (c)CG1309S319A*

3.8 Competitive ABPP

A competitive ABPP shows that in vitro expressed CG15111 and CG1309 has been inhibited by mammalian orthologs inhibitors Tetrahydrolipstatin (THL) and KC01 respectively (Fig 3.22 a, c). THL can also inhibit CG1309 (Fig 3.22 b).

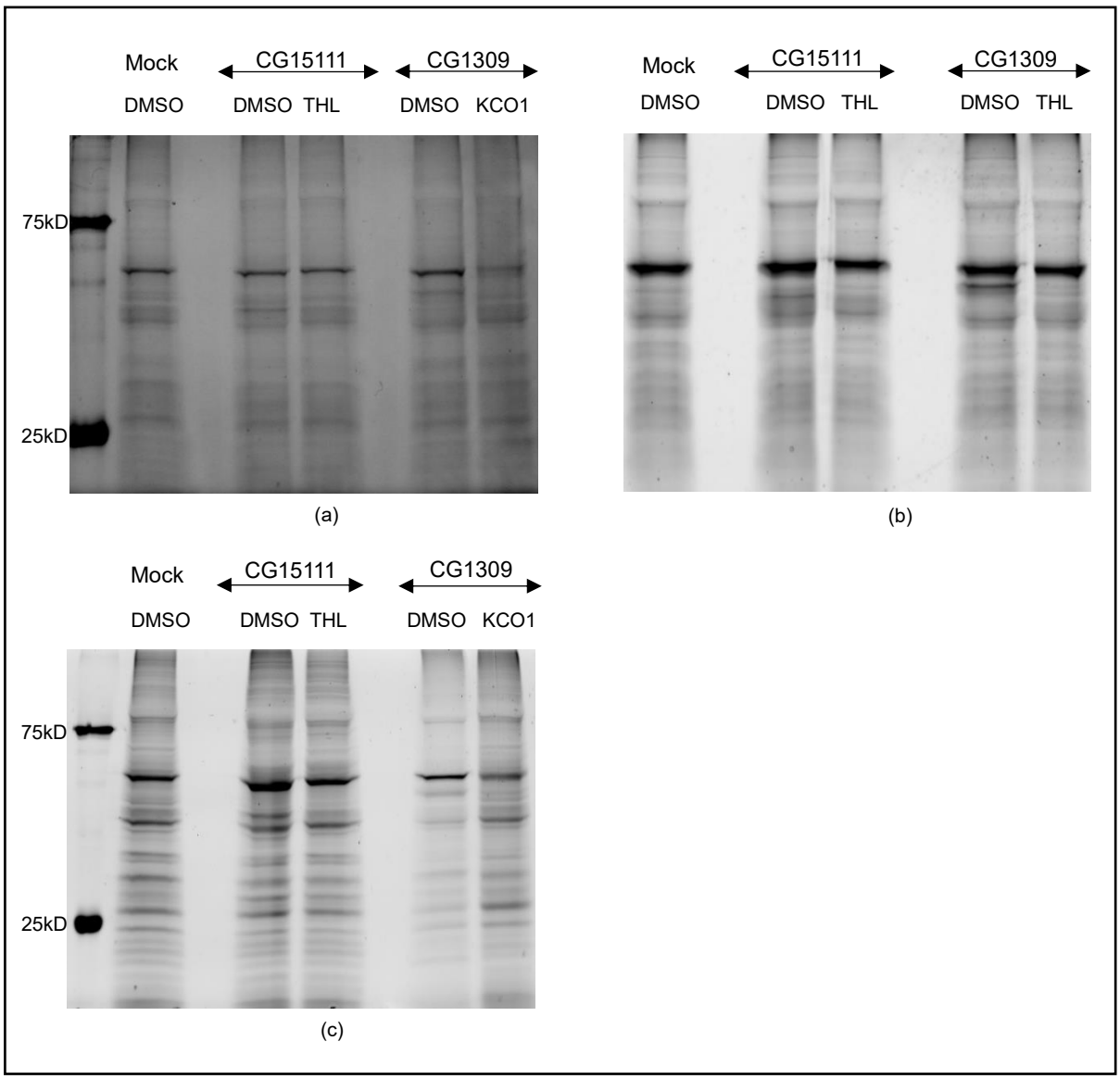


Figure 3.22 Competitive ABPP_*(a) CG1511-THL, CG1309-KC01 (b) CG15111-THL, CG1309-THL (c) CG15111-THL, CG1309-KC01*

3.9 Bacterial Clearance Test (BCT)

BCT shows CG15111 was able to clear out the bacterial infection Fig 3.23.

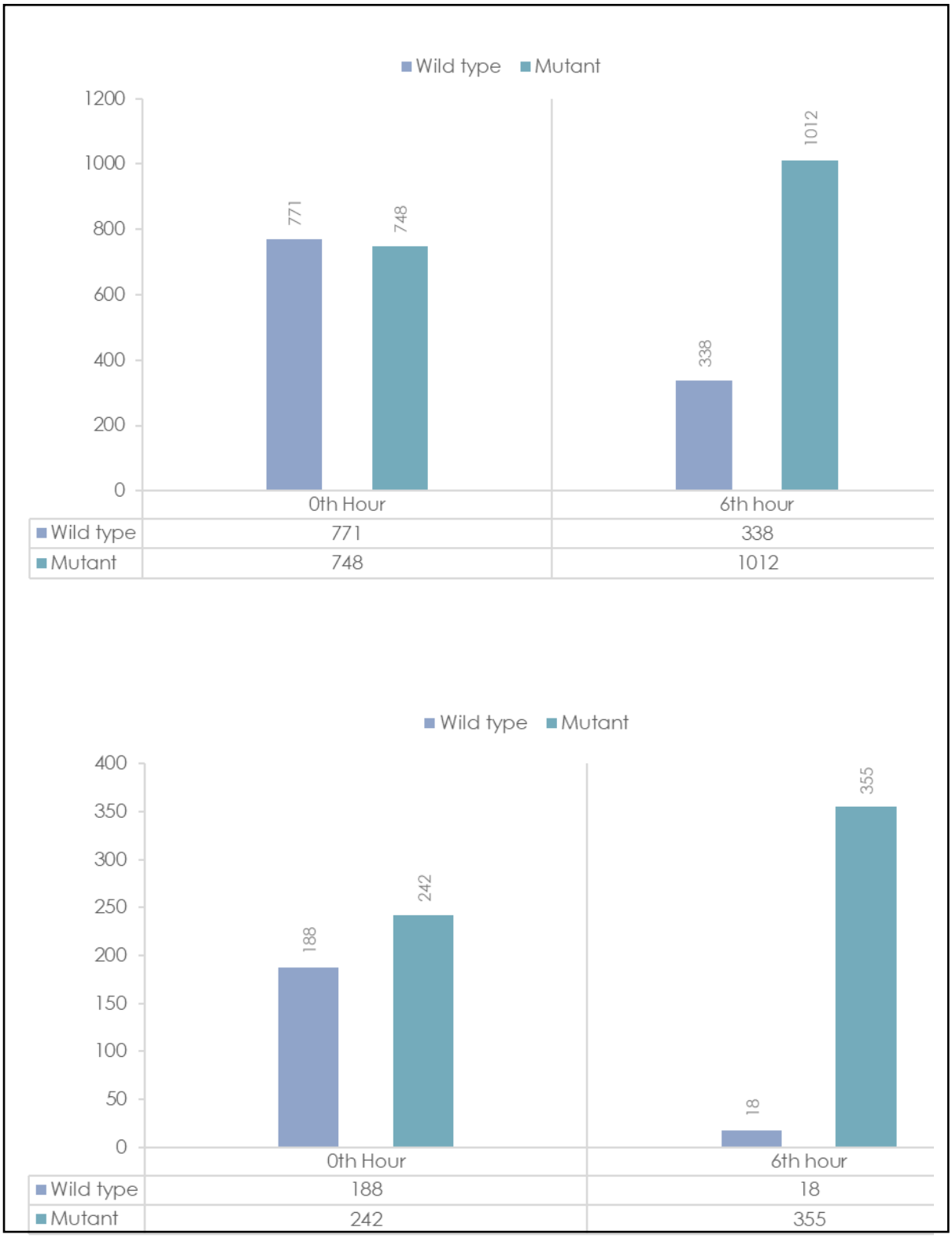
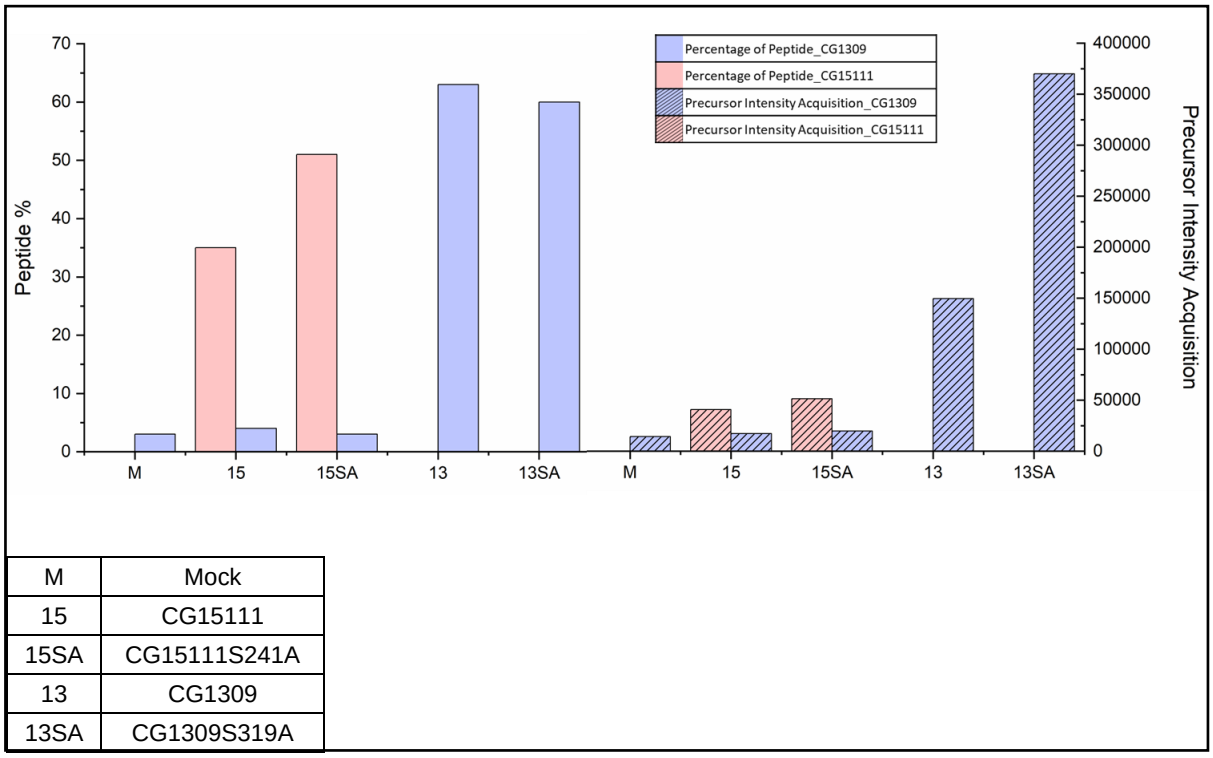


***Figure 3.23 BCT**_graph showing CG15111 was able to clear out bacterial infection with time*

3.10 Mass Spec Results

LC-MS analysis of in vitro expressed N terminal 6x His tag shows there is a successful protein expression Fig 3.24.



M	Mock
15	CG15111
15SA	CG15111S241A
13	CG1309
13SA	CG1309S319A

Figure 3.24 The Mass spec data_*showing that there is a successful expression of N terminal 6x His tag plasmids.*

4. CHAPTER 4 _DISCUSSIONS

4.1 Phospholipases in *Drosophila melanogaster*

The fly model for the disorder PHAC is the primary aim of my project. In order achieve the same we have done some data digging, couple of experiments and their analysis. The initial step was to identify the phospholipases in the Drosophila. Phospholipids are coming under the enzyme class *Serine hydrolase* and all the serine hydrolase in the *Drosophila melanogaster* has been already identified(Kumar et al., 2021). Using that data available and the fly base we have been classified the phospholipases in drosophila into different categories based their site of cleavage. The different subgroups have their own enzyme classification number (EC number) which is given based on the reaction they catalyze. We found 34 phospholipases in *Drosophila melanogaster* and the have been classified under the sub-groups which are *Phospholipase A1 (EC 3.1.1.32), Phospholipase A2 (EC 3.1.1.4), Phospholipase B (EC 3.1.1.5), Phospholipase C (EC 3.1.4.3 & 3.1.4.11), Phospholipase D (3.1.4.4) and others* (Table 3.1). Apart from these EC numbers there are some other sub categories comes under them which having a different EC number like phosphoinositide phospholipase B. Most of these genes are not yet characterized yet and we only have predictions on these genes using different parameters. As per the fly base data, among the categorized enzymes 15 has their orthologous genes have phospholipase activity. Using the fasta data collected from National center for biotechnology information (NCBI) generated a phylogenetic tree using neighbor joining method in megaX software Fig (3.1). The phylogenetic tree shows an interesting trending that there is high sequence similarity between PLA1s and we have observed 2 subgroups there. To understand this trend, we have tabulated the cytogenetic map of these 34 phospholipases (Table 3.2) and we have observed that those genes are located nearby in the same gene. From these data we can make a hypothesis that this high sequence of PLA1 might arise due to the recent gene duplications.

4.2 Fly genes and their Mammalian Orthologs

Among these 34 phospholipases our interests are CG15111 and CG1309 which are orthologous to mammalian ABHD12 and ABHD16A. Using Clustal omega multiple sequence alignment it's found that CG15111 has a sequence similarity of 36.8% and 37% with human and mice ABHD12. Similarly, CG1309 has a sequence similarity of

41% and 40% with human and mice AHD16A and they also have conserved serine residue in their active site. We have checked the transmembrane domains of CG15111 and CG1309 using Using TMHMM - 2.0 and the predicted transmembrane domains are similar to their orthologues ABHD12 and ABHD16A. Even though they don't have exact same transmembrane domains it's found that ABHD12 and CG15111 have one transmembrane domain and both have same length and same observed for ABHD16A and CG1309 but they have two transmembrane domains (Fig 3.3).

4.3 Mammalian Antibodies in Fly Proteins

Since we have observed these similarities between the mammalian gene and fly gene, we have tried to find out that do mammalian antibodies have any affinity towards the fly proteins. We have taken larva (3rd instar) and early pupa and lysed with pestles in 100µL PBS and loaded on 4% SDS stacking gel with loading dye at 80v then resolved in 10% SDS gel at 110v. Then further transferred at the conditions mentioned in section 3.3. The primary antibody used is His-Probe mice monoclonal igG$_1$ with a dilution of 1:1000 in 5% milk in 0.1% TBST and the secondary antibody is αM-HRP with a dilution of 1:10000 in 5% milk in 0.1% TBST. The results were suggesting that the mammalian antibodies don't have any affinity towards the fly protein as we observed random binding with ABHD12 antibody and ABHD16A antibody doesn't show any binding at all.

4.4 SLiCE Cloning and *In Vitro* Expression in S2 Cells

To study more about these genes and characterizing them, they need to be *in vitro*. We have planned to express them in the S2 cells using pRM as a vector with a 6x His tag in the N terminal using SLiCE cloning technique. We have PCR amplified the gene of interests (CG15111 and CG1309) with 6x His tag in the N terminal from the cDNA given by Kundan using the designed primers (primer 1 & 2 for CG15111 and primer 3 & 4 for CG1309) mentioned earlier (Table 2.2 List of primers), also the pRM vector with the primers 9 & 10. Next, we have generated a serine to alanine mutant version of these genes with the mutagenesis primers mentioned in the list of primers table (primer 5&6 and primer 7&8) and further amplified with wildtype primers for amplification correspondingly. The serine located in the 241th position was mutated to alanine and CG15111^{S241A} mutant was generated and similarly CG1309^{S319A} was generated from CG1309. The serine to alanine mutant was preferred because alanine

being a non-reactive group and structural similarity, these 2 features make the alanine the perfect selection of mutant generation. All the PCR products were analyzed by agarose gel electrophoresis and the results were showing positive results as the final size of CG15111 after addition of tag was 1266 and CG1309 is 1605 bp. All the genes were further cloned to S2 cells expressing pRM vector using SLiCE technology as mentioned earlier and all clones were analyzed in agarose gel. We have done a colony PCR screening with forward and reverse primers for both genes and the PCR product was showing proper bands indicating cloning was successful as our gene of interest is the only binding site for primers. For Sanger sequencing diluted the plasmid into 20 µL of 100ng/µL concentration and sequenced with designed sequencing primers at Biokart India Pvt limited. The sequencing results have confirmed that all genes were successfully cloned in to pRM vector. These plasmids were further transformed into DH5α for obtaining higher concentration and can be used for transient expression in S2 cells for further studies.

For expressing in S2 cells, all plasmids were diluted to 1µg/µL concentration. Using CG15111 we have tried to standardize the transient transfection with CASPR, a soluble protein, as a positive control. The initial variable we tried to fix is the ratio of TransiT2020 to DNA. We have tried 2:1, 3:1 and 4:1 ratio (kept the CG15111 volume as 1µL) and CASPR with a 2:1 ratio. The incubation time was 24 hours. After the transfection products were harvested and analyzed with western blot with the conditions mentioned in fig 3.8 and the results suggests that transfection was not successful. Since the CASPR was expressed well the western blot protocol used was fine. To troubleshoot the problem, we have repeated the experiment with all cloned plasmids with 2:1 ratio and 24 hours incubation. The western blot result was the same that CASPR expressed well and all the cloned plasmids were not expressed (fig 3.9). The possible reasons for the experimental failure might be a problem in the cloned plasmids or the transfection conditions were not optimum for the cloned plasmids (CASPR and cloned plasmids might require different reaction conditions). To nullify the hypothesis that not showing expression was due to defect in cloned plasmids we have repeated the transfection and the samples were analyzed in western blot and ABPP (fig 3.10 a, b), the ABPP will detect the activity of serine hydrolase rather than detecting the 6x His tag. The western blot still didn’t show any positive bands but surprisingly it is observed that the ABPP image was showing a positive band as both

wild types have bands in proper size and the mock and mutants were devoid of that which indicates the successful transfection. The ABPP analysis confirmed that the cloned plasmids are working fine and the problem lay in something else. Since the CASPR was expressing well the antibodies were working fine and through ABPP we found the plasmids are also good. Through this western blot we are trying to detect the 6x His tag in the cloned plasmids. So, we hypothesized that there is a possibility that somehow the N terminal 6x His tag is getting chopped off and that might be the reason for no bands in the western blot. As a step to nullify this problem we have decided to generate C terminal tagged clones. Mean time we have optimized the incubation time for better ABPP analysis and the found that with 72 hours of incubation the expression was higher but 24 and 48 hours also gives significant results (fig 3.12). We also checked the affinity of mammalian antibodies towards in vitro expressed proteins and the result was similar to the fly protein analysis (fig 3.13) as CG15111 has random binding and CG1309 had no binding at all.

The C terminal 6x His tagged pRM-CG1309 was generated for further studies and the generation of plasmid was through SLiCE protocol and confirmed through sequencing. The transfection also followed the same protocol with 2:1 ratio and 24 hours incubation. The harvested products were analyzed using western blot and ABPP (fig 3.16 a, b). The western blot showed result was not satisfactory as we didn't get a proper band in there but the ABPP was successful as we obtained a proper band which is absent in mock. To optimize the western blot, we have varied the transfer conditions and the new transfer conditions were 80v for 3-4 hours at 4°C. To ensure the successful transfer we have treated the transfer membrane with Ponceau Red (further washed with 0.1% TBST to remove the dye) and the gel with Coomassie brilliant blue. The western blot analysis still was negative and ABPP was giving positive bands (fig 3.17 a, b). We repeated the same with experiment with a different antibody, instead of His probe Mice monoclonal igG1 we used His probe rabbit polyclonal igG1, with the previous western blot conditions. We have obtained a positive band in C terminal 6x His tagged pRM-CG1309 plasmid (fig 3.18), which suggest that the mono clonal antibody has some defect which is further confirmed in our lab by other lab members. Unfortunately, the we were unable to reproduce the positive results that obtained with the poly clonal antibody. Since this issue persisted, we tried to add another tag instead of 6x His tag.

4.5 Plasmids with a C Terminal FLAG Tag

To overcome the issues with anti-His antibodies we have tried to add FLAG tag to the plasmid at the C terminal of gene. We have designed one forward and two reverse primers for each gene. One reverse primer will help to add the FLAG tag to the gene and the other one will make the gene ends homologous to pRM vector. We have amplified the genes and pRM vector (fig 3.19) followed by the SLiCE cloning. The successful cloning was confirmed through colony PCR (fig 3.20) and Sanger sequencing (fig 3.21). We have generated all clones except pRM-CG15111 and the generated plasmids can be used for transient expression in S2 cells.

Parallel to this we have also carried out a LC-MS based validation of cloned plasmids and the data was so satisfactory as it indicated that all the four plasmids were expressed (fig 3.24). We have observed a very minute endogenous expression of CG1309 in S2 cells and CG15111 is not at all expressing in the mock. The plot confirms that all the plasmid expression was detected in the LC-MS which is conclusive evidence for successful in vitro expression of cloned plasmids.

4.6 Competitive ABPP Analysis

Inhibitors play important role in characterizing a protein and finding the optimum inhibitor is essential for our studies. The mammalian orthologue inhibitors are already known and initially we checked that whether they have any effect on these proteins. The inhibitors used were THL (Tetrahydrolipstatin) and KC01. THL is a general lipase inhibitor which can inhibit a set of lipase enzymes with some exceptions and KC01 is specialized inhibitor for ABHD16A. We have used ABPP to analyze the reaction and found that mammalian orthologue's inhibitors can also inhibit the fly protein (fig 3.22). Since KC01 being an expensive inhibitor, we have also checked the inhibition capacity of THL towards CG1309 and found that THL can also inhibit CG1309 in good manner. These enzymes can be used further for unfolding the mechanism and charterers of these enzymes.

4.7 Effect of CG15111 in Fly Immunity

Immunity is always a prime concern in every organism and those genes that have effect in immunity has a high importance. We have tried to find that CG15111 has any

role in the fly immunity. Bacterial clearance test was assigned to found this characteristic. We have taken CRIMIC mutant of CG15111 and wild type flies for the analysis. The BCT shows that the wild type was able to clear out the bacterial infection after a particular time which was lacking in the mutant. We have done 2 replicates for these experiments and final conclusion remained same. In the first replica the 0^{th} hour plates have 771 and 748 bacterial colonies in wild type and mutant respectively and the 6^{th} hour plate of wild type has only 338 colonies and the in the mutant failed to clear out the bacterial infection as the number of colonies got increased to 1012. Similarly, the 2^{nd} replica also had a change from 188 colonies to 18 colonies for wild type and mutant got changed from 242 to 355 colonies. The data implies the CG15111 has some role in immunity which clears the bacterial infection which ability will compromised if CG15111 genes is mutated.

We have generated C terminal FLAG tagged cloned and they can be used for further studies. We got into a conclusion on His tagged plasmids that, they were expressing well and due to the defective antibody and some cleavage happening in the N terminal it is not visible through western blot. To confirm the scenario in the N terminal, upon successful expression of C terminal FLAG tagged plasmids we can try to add a N terminal FLAG tag and confirm. If the N terminal cleavage is confirmed it is also a question rising that the *do the N terminal cleavage has any significant impact on the activity of these genes*. The substrate for these genes is yet to found upon successful transfection followed by substrate assay we can get an answer to this. We have started this project with a hypothesis that these genes are phospholipases as per fly base predictions and the fly base predicts these genes might have other function. So, substrate assay can bring a conclusion on these. The further characteristics of these genes are yet to be unfolded.

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