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SUBCLONING, EXPRESSION, AND ENZYMATIC STUDY OF PRMT5

by

RAN GUO

Under the Direction of Yujun George Zheng

ABSTRACT

Protein arginine methyltransferases (PRMTs) mediate the transfer of methyl groups to arginine residues in histone and non-histone proteins. PRMT5 is an important member of PRMTs which symmetrically dimethylates arginine 8 in histone H3 (H3R8) and arginine 3 in histone H4 (H4R3). PRMT5 was reported to inhibit some tumor suppressors in leukemia and lymphoma cells and regulate p53 gene, through affecting the promoter of p53. Through methylation of H4R3, PRMT5 can recruit DNA-methyltransferase 3A (DNMT3A) which regulates gene transcription. All the above suggest that PRMT5 has an important function of suppressing cell apoptosis and is a potential anticancer target. Currently, the enzymatic activities of PRMT5 are

not clearly understood. In our study, we improved the protein expression methodology and greatly enhanced the yield and quality of the recombinant PRMT5. In addition, mutagenesis and enzymatic studies implicate an interesting mechanism of PRMT5 activity regulation.

INDEX WORDS: PRMT5, Histone, Radioactivity, Mutagenesis, Protein purification

SUBCLONING, EXPRESSION, AND ENZYMATIC STUDY OF PRMT5

by

RAN GUO

A Thesis Submitted in Partial Fulfillment of the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2010

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Ran Guo
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	ix
LIST OF FIGURES.....	xii
LIST OF ABBREVIATIONS.....	xvi
Chapter	
1. Background and Introduction.....	1
2. Experimental Methods.....	9
2.1 PCR Reaction to Amplify Target Gene.....	9
2.2 Gel Extraction with Q1Aquick Gel Extraction Kit Protocol.....	10
2.3 Restriction Digestion for Cutting the Target Gene and Vector.....	11
2.4 DNA Ligation of Gene and Vector.....	12
2.5 Transformation for Using Bacteria to amplify Constructed Plasmids.....	12
2.6 Expression of His-tagged Protein.....	14
2.7 Radioactive Assay for Activity Test.....	17

2.8 General Radioactive Assay Text.....	18
2.9 Quickchange to Make Truncated PRMT5 Gene/Protein.....	19
3. Subcloning of PRMT5 Gene into an Expression Vector.....	21
3.1 Subcloning PRMT5 into PGEX4T1.....	21
3.1a PCR Experiments.....	21
3.1b Restriction Digestion of PRMT5 and pGEX4T1.....	22
3.1c DNA Ligation to Rebuild the Plasmids.....	23
3.1d Transformation of the Recombinant Plasmid into XL1 Blue Bacteria....	24
3.2 Subcloning shPRMT5 into pTXB1 and pET28a.....	26
3.2a Preparation shPRMT5 from puc57-shPRMT5 Plasmid.....	26
3.2b Insertion of shPRMT5 into pTXB1 and pET28a.....	27
3.3 Subcloning shPRMT5 into pTXB1.....	30
4. Expression of His-Tagged shPRMT5 Protein	32
5. Enzymatic Activity of shPRMT5.....	35
5.1 Radioactive Assay of His-tagged shPRMT5.....	36
5.2 Degradation of shPRMT5.....	37

5.3 Radioactive Assay of Degraded shPRMT5.....	37
5.4 FPLC of shPRMT5.....	40
5.5 Radioactive Assay of Different Protein from shPRMT5 on H4-20.....	41
6. Mutagenesis of shPRMT5.....	46
6.1 Quickchange Reaction to delete 1-50 Amino Acids at the N-terminal End of shPRMT5.....	46
6.2 Quickchange Reaction to delete 1-200 Amino Acids at the N-terminal End of shPRMT5	48
7. Expression and Enzymatic Test of Truncated shPRMT5.....	50
7.1 Expression of Truncated shPRMT5 in BL21 (DE3).....	50
7.2 Radioactive Assay of Truncated shPRMT5.....	51
8. Bioinformatic Study of PRMT5.....	55
8.1 Blast Search of PRMT5.....	55
8.2 CLUSTAL W2 analysis of PRMT5, PRMT1, PRMT3 and CARM1.....	56
8.3 Pattern VLMVLGAGRGPLV in Prosite Server to Figure out the SAH/SAM Binding Site Position.....	56
8.4 Predict the Structure of PRMT5 SAH/SAM Binding Site.....	57
8.5 An Analysis SAH Binding Site of Predicted Structure of PRMT5 Using AutoDock.....	58

9. Conclusions and Discussion.....60

References.....62

LIST OF TABLES

Table 1. Composition of the PCR reaction samples.....	10
Table 2. Composition of the digestion reaction of target gene and vector.....	12
Table 3. Component of Bradford protein concentration system measurement.....	16
Table 4. Composition of the radioactive assay in different substrate concentrations	18
Table 5. Composition of the general radioactive assay.....	19
Table 6. Quickchange PCR reaction system.....	20
Table 7. Quickchange PCR Program.....	20
Table 8. Calculations of the concentration and molecular weight (MW) of PRMT5, pTXB1 and pET28a.....	27
Table 9. Setup of ligation reaction.....	28
Table 10. General test of shPRMT5 activity on H4-20 and H3-20.....	35
Table 11. Results of activity assay of shPRMT5 on H4-20 and H3-20.....	35
Table 12. Results of duplicate activity assay of shPRMT5 on H4-20 and H3-20.....	36

Table 13. Compositions Composition of additional general radioactive assay of shPRMT5 on H4-20 and H3-20.....	38
Table 14. Results of additional radioactive assay of shPRMT5 on H4-20 and H3-20.....	38
Table 15. Composition of radioactive assay of different sections of shPRMT5 on H4-20.....	42
Table 16. Results of the five sections of shPRMT5 activity on H4-20 methylation	42
Table 17. Results of the five sections of shPRMT5 activity on H4-20 methylation	43
Table 18. Results of the activity of 5 group normalized concentrations on H4-20.....	43
Table 19. Composition of radioactive assay of truncated of shPRMT5 on H4-20.....	51
Table 20. Results of general test of radioactive assay of truncated PRMT5.....	52
Table 21. Composition of radioactive assay of truncated of shPRMT5 on H4-20.....	52

Table 22. Results of duplicate general test of radioactive

assay of truncated PRMT5.....53

LIST OF FIGURES

Figure 1. Reaction of PRMT enzyme catalyzed arginine methylation	2
Figure 2. The domain structure of PRMT proteins.	3
Figure 3. Nucleosome structure and posttranslational modification.....	4
Figure 4. Mechanism of PRMT5 and DNMT3A cooperation for inhibition of transcription of the target gene	7
Figure 5. Process and composition of radioactive assay	18
Figure 6. PCR products of PRMT5 DNA	22
Figure 7. Checking the ligation efficiency of PRMT5 efficiency with restriction digestions.	24
Figure 8. Alignment of two sequences for checking the identity	25
Figure 9. SDS-PAGE of expressed of GST-tagged PRMT5.....	25
Figure 10. Band A is unclear. Band B are puc57-shPRMT5 plasmid. Band C is puc57 vectors. Band D is unclear. Band E is shPRMT5	27

Figure 11. DNA bands on a 1% agarose gel for checking the ligation efficiency of shPRMT5 and pTXB1.....	29
Figure 12. DNA bands on a 1% agarose gel for checking the ligation efficiency of shPRMT5 and pET28a	29
Figure 13. DNA bands on a 1% agarose gel for checking the ligation efficiency of the second sub-cloning attempt of shPRMT5 and pTXB1.....	31
Figure 14. 12% SDS-PAGE with supernatant, pellets, washing solution 1, 2, 3, 5, 7 samples in upper gel	33
Figure 15. 12% SDS-PAGE with shPRMT5 protein. It seems that PRMT5 has degraded into two major bands at around 60 kD and 25 kD	34
Figure 16. Radioactivity results for shPRMT5 to H4-20 and H3-20.....	36
Figure 17. 12% SDS-PAGE Gel representation of the shPRMT5. shPRMT5 degraded further and generated multiple bands	37
Figure 18. Radioactivity results for shPRMT5 on H4-20 and H3-20.....	39
Figure 19. FPLC settings.	40

Figure 20. 12% SDS-PAGE gel with different groups of proteins with different molecular weights separated by FPLC	41
Figure 21. Activity of 5 fractions of the shPRMT5 and original shPRMT5.....	44
Figure 22. 12% SDS-PAGE gel showing the samples after condensation of the different groups of protein and the previous shPMRT5 as the control	44
Figure 23. Quickchange with PCR reaction products of 51-637 amino acids of PRMT5.....	47
Figure 24. Quickchange with PCR reaction products of 201-637 amino acids of PRMT5.....	49
Figure 25. 12% SDS-PAGE gel showing 51-637 and 201-637 protein after purification.....	51
Figure 26. The CPM values of 51-637, 201-637, full-length shPRMT5 and negative control of shPRMT5.....	53
Figure 27. BLAST analysis of PRMT5.....	55
Figure 28. CLUSTAL W2 analysis of PRMT5, PRMT1, PRMT3 and CARM1.....	56
Figure 29. SAH binding site in 3D PRMT1 structure	57

Figure 30. PRMT5 SAH binding site position in the prediction structure which use

PRMT1 as a template58

Figure 31. The arrow pointing to the best docking site of SAH in the prediction

structure of PRMT5.....59

LIST OF ABBREVIATIONS

IPTG	Isopropyl β -D-1-thiogalactopyranoside
DTT	Dithiothreitol
TAE	Tris-acetate-EDTA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shPRMT5	Synthetic human PRMT5
EtOH	Ethyl alcohol
PMSF	Phenylmethanesulfonyl fluoride

Chapter 1 Background and Introduction

PRMTs (protein arginine methyltransferase) are a group of proteins in mammalian and plant cells. PRMTs use S-adenosylmethionine (AdoMet/SAM) as a methyl donor in the cells (1) (2). Different PRMT enzymes share a core conserved region which is around 310 amino acids long that is involved in the enzymatic reaction. In addition, PRMTs can catalyze the methyl transfer through various ways to the nitrogen of the terminal guanidine of arginines. Firstly, as shown in Figure 1, it can transfer a single methyl group to the terminal guanidino nitrogen, namely, monomethylated arginine (MMA). Secondly, it can transfer two methyl groups symmetrically to the two nitrogens of the guanidine to form symmetrically dimethylated arginine (SDMA). Lastly, it can transfer two methyl groups asymmetrically to the same nitrogen of the guanidine to form asymmetrically dimethylated (ADMA) (3).

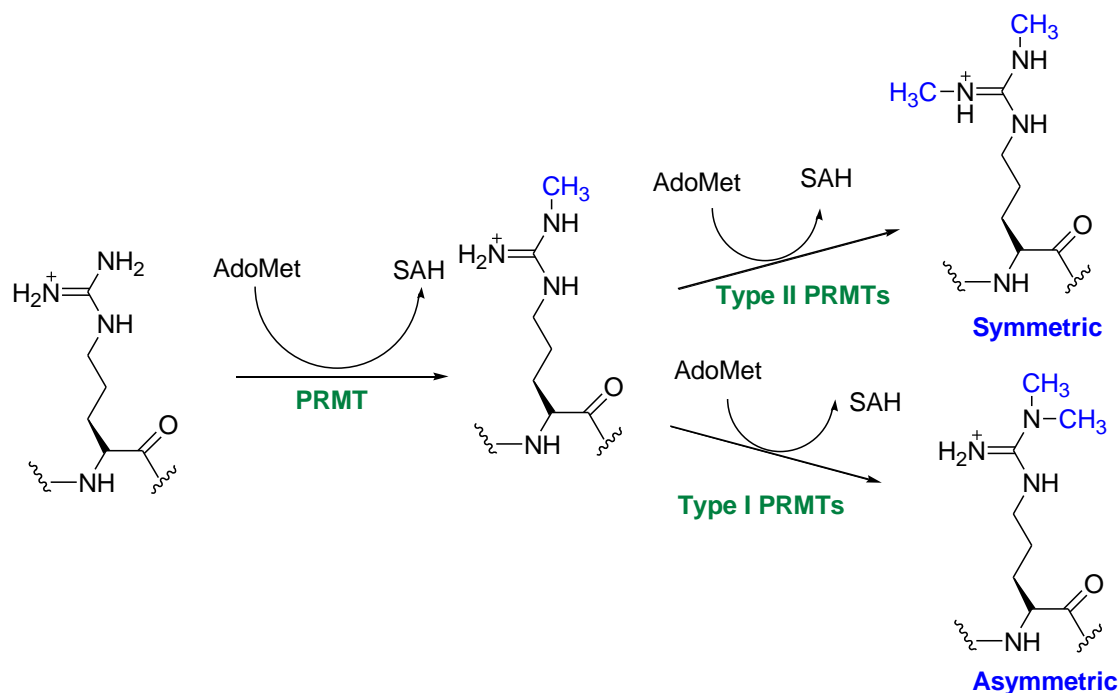


Figure 1. Reaction of PRMT enzyme catalyzed arginine methylation.

Because differences in arginine methylation leads to different functions (4), PRMTs are divided into two types according to whether the second methyl group was asymmetrically (type I) or symmetrically (type II) added to the nitrogen of arginine as shown in Figure 1. Type I includes PRMT1, PRMT2, PRMT3, PRMT4, PRMT6, PRMT8 and Type II includes PRMT5, PRMT7, PRMT9, PRMT10 and PRMT11 (3). PRMTs share a highly conserved domain which is shown in Figure 2. Additionally, some PRMTs contain other specific motifs, such as SH3, zinc finger, etc.

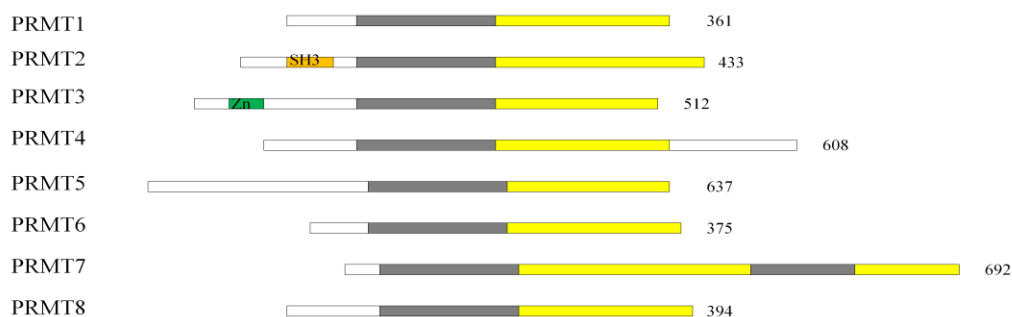


Figure 2. The domain structure of PRMT proteins. Gray part indicating the highly conserved PRMT core region, green part indicating the zinc finger in PRMT3 and orange part indicating the SH3 domain in PRMT2 (5).

PRMTs belong to a type of posttranslational modification enzymes which transfer methyl groups to the arginine of histones to regulate gene expression (6). Chromatin is composed of DNA and histone proteins in which DNA wraps around histones called nucleosomes, which can be further packed to form chromosomes. Figure 3 illustrates some posttranslational modification sites on the histone tails of nucleosome. Chromatin regulates gene expression through modification of histones, which can be altered by different histone modifying enzymes such as histone acetyltransferase and methyltransferase (3). In histone methylation, the methyl group transferred usually occurs on arginine and lysine residues, which can change the chromatin structure to inhibit or stimulate gene transcription. The histone core contains four major highly conserved histones, H2A, H2B, H3 and H4 with very similar three-dimensional structure, and a linker subunit H1 that is located of where DNA enters and leaves (7) (8). Two H3 and H4 form a tetramer and combine with two H2A-H2B dimers to construct the histone octamer. The left-handed super-helix DNA wraps around the histone octamer (9), so it can be highly compacted to store long double-stranded DNA into the small nucleus. The histone core wound by supercoiled DNA is the main part of nucleosomes. Every histone has an amino-terminal tail which extends from the histone core structure. The H3 and H4 tails contain some lysine and arginine residues

that can be methylated to modify the interaction between the histone and DNA (10). H3R8 and H4R3 are well-known substrates of PRMT5 (11) (12), and H4R3 can also be methylated by PRMT1 (13). Therefore, to study PRMT5 enzymatic properties, H3R8 and H4R3 are feasible researching element.

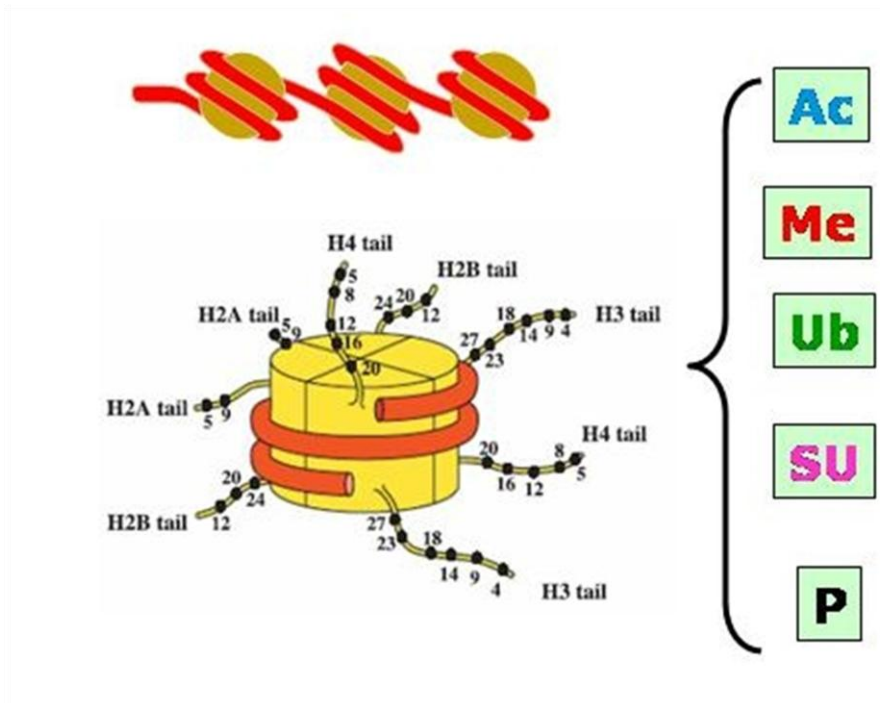


Figure 3. Nucleosome structure and posttranslational modification.

In leukemia and lymphoma, PRMT5 was shown to suppress the Retinoblastoma (RB) family of tumor suppressors through methylating H3R8 and H4R3 at the transcriptional level (14). Additionally, PRMT2, which catalyzes the asymmetric dimethylation of arginine, also interacts with the RB protein through the AdoMet-binding domain to form complexes with E2F (15). E2F is a transcription factor that regulates the cell proliferation-related gene expression (16). p53 is a critical protein involved in apoptosis of tumors which respond to DNA damage. However, PRMT5 as a cofactor of p53 can methylate p53 to regulate p53 protein interaction with target genes through the modification of the promoter of p53 (17) (18). In addition, knockdown of

PRMT5 can induce G1 arrest, and consequently inhibit of cell proliferation (19). Through methylation of H4R3, PRMT5 enzyme has an important function in suppressing cell apoptosis.

A zinc finger is an amino acid sequence motif that interacts with one or several zinc ions to maintain its structure. A zinc finger usually consists of an α helix and a β sheet and coordinates with two cysteine and two histidine residues. A KRAB-containing zinc finger is a critical DNA binding protein with Kruppel-like associated box (KRAB) at the beginning. ZNF224 is a member of KRAB-ZFP family which includes 19 zinc finger motifs at the C-terminus. PRMT5 can physically coordinate with ZNF224 to form a complex which will be recruited to the L-type aldolase A promoter to suppress transcription around H4R3 (20). Additionally, Ajuba is a protein that can repress the zinc finger-protein Snail by recruiting PRMT5 to form complex to silence the Snail target gene. NR2 (nuclear-receptor binding motifs), which is necessary for binding PRMT5, is one of the putative nuclear-receptor binding motifs which exist in Ajuba. Furthermore, PMRT5 was observed to bind to the NR2 motif of Ajuba, which is also the binding site of RAR (retinoic acid receptors), to inhibit Ajuba/RAR interaction. Thus, PRMT5 seems to be like a “switch” of Ajuba which can interact with Snail or RARs (21).

In addition, PRMT5 can also generate symmetric dimethylarginine in non-histones proteins. pICln is a 26 kDa protein which can bind to Sm protein in human cells to regulate the RNA-splicing. By interacting with the pICln, PRMT5 can stimulate symmetrical dimethylation of the RG tail which is rich with arginine and glycine at the C terminal end of the Sm protein. However, this phenomenon inhibits PRMT5 methylation function of histones (22). Other tumor suppressors could also interact with PMRT5. Furthermore, a cooperator of PRMT5 (COPR5) was reported to be involved in the function of PRMT5 in vitro and in vivo. COPR5 binds to PRMT5 tightly and specifically, which helps to methylate H4R3. COPR5 and PRMT5 complex

regulates the promoter of cyclin E1 (CCNE1) and COPR5 interacts with the amino terminus of H4 to play a critical role between PRMT5 and the target gene (23). This reveals that PRMT5 has an additional function of maintaining the structure of certain proteins with methylated arginine. In addition to GM130, ribosomal protein s10 (RPS10) was reported as a substrate of PRMT5. PRMT5 catalyzes the methylation of Arg 158 and Arg 160 of ribosome protein RPS10 to regulate proliferation of the cells (24). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has activity of selective antitumor suppressing tumors. However, PRMT5, as a TRAIL receptor-binding protein, binds to receptor 4 and receptor 5 of TNF selectively. Additionally, PRMT5 can contribute to nuclear factor-kappaB (NF-kappaB) which resistance TRAIL pathway to inhibit tumor cell necrosis (25). So, PRMT5 prefers to combine with other protein to regulate target gene indirectly.

CpG sites consist of cytosines which are next to guanines connected by a phosphodiester bridges. CpG sites are often located in the promoters of genes. The cytosine of CpG sites can be methylated by DNA methyltransferase to 5-methylcytosine that can silence genes. This reaction is an epigenetic modification function (26) (27). In addition, previous research showed that methylated H4R3 by PRMT5 can recruit DNMT3A which is one of DNA methyltransferases to methylate promoter CpG islands to silence gene transcription (28) (29). Figure 4 illustrates the mechanism of PRMT5 and DNMT3A regulating function. Therefore, PRMT5 and DNA methyltransferases might have a coordinated function to mediate gene expression. H4R3Me₂ modified by PRMT5 can support a target for DNMT3A binding to methylate CpG sites. On the other hand, methyl-CpG-enriched region also can recruit PRMT5 complex to repress transcription. This shows a cooperative function in regulating gene expression at an epigenetic level (30).

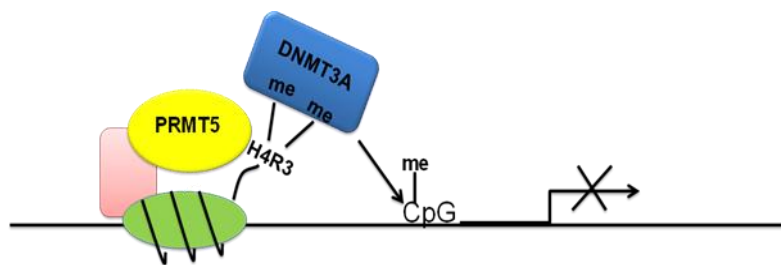


Figure 4. Mechanism of PRMT5 and DNMT3A cooperation for inhibition of transcription of the target gene.

It also shows that the ribosomal RNA gene promoter is regulated by methylated H4R3 and H3R8 which means PRMT5 not only can regulate RNA polymerase II, but also RNA polymerase I. H3R8Me2 and H4R3Me2 generate different pathways for suppressing the ribosomal RNA gene transcription in resting B cells and HeLa cells. Hereby, PRMT5 inhibiting function can operate in some different genes (11). Histone methylation can not only generate DNA damage, but also repress transcription (31). Methylation of CpG-rich sequence will prevent transcription factors from binding, which can play an active role in transcription in the genes (32) (33). However, another hypothetical pathway to inhibit the gene transcription through DNA methylation is opposite. Other than repelling the factors, methylated CpG sites can recruit methyl-CpG-binding domain (MBD) family protein which includes MeCP2 (34), MBD1, MBD2, MBD3, MBD4 and KAISO without an MBD domain (35). These proteins were observed to bind methylated DNA and associate with different corepressor to inhibit gene transcription (36). Therefore, PRMT5 and DNA methyltransferase have a relative function to methylate histone and DNA to silence the gene transcription at epigenetic level.

Finally, PRMT5 inhibits protein transcription. These proteins usually have direct or indirect function in tumor cell suppression. Moreover, ribosomal RNA synthesis by RNA polymerase I is also influenced by PRMT5 (11). Meanwhile, PRMT5 usually associate with other protein to form a complex, which can be recruited to promoter regions of target genes. Then the

symmetrical dimethylation occurs on arginine H4R3 and H3R8 of histones which recruits DNMT3A to the promoter and then methylates CpG sites to inhibit transcription. This is the hypothetical pathway of PRMT5 suppression of gene transcription.

Chapter 2 Experimental Methods

2.1 PCR Reaction to Amplify Target Gene

A PCR reaction was set up to amplify the PRMT5 gene. Through PCR, the PRMT5 genes can be amplified to produce many copies of it for future experiments. Primers are designed around 12-16 nucleotides which were complementary to each 3' strand of the DNA templates. The PCR reaction includes the DNA templates, primers, buffers, dNTP, DMSO, MgSO₄, and DNA Native pfu polymerase, which are required in the system and the volumes of each component are shown in Table 1. The first step is the denaturation with the temperature set at 94°C for 45 seconds to denature the double stranded DNA by breaking the hydrogen bonds between the bases. Next, the temperature is decreased to 55°C for 45 seconds to let the primers anneal to the DNA templates which is called the annealing step. In this step, new hydrogen bonds form between primers and single strand DNA templates and DNA polymerase binds to the complex to start DNA replication. The temperature is then lifted to 72°C for 3 minutes to make the DNA polymerase active to elongate the DNA copies. The system then cycles back to step one and repeats this 25 times. After the last cycle, the system is set to 72°C for 10 minutes to make sure the copies of DNA are completely extended and it is then held at 4°C. PCR is the best way to get lots of DNA from a very small amount efficiently and precisely. When the PCR reaction is finished, the products will be confirmed as the correct DNA size by gel electrophoresis. The 1% agarose gel was composed of 300 mL of 1X TAE buffer and 3 g of agarose. To dissolve the agarose into the 1X TAE buffer, it was heated in a microwave oven for 4 minutes. 8 µL of ethidium bromide (EB) which can intercalate with DNA fragments and shows orange

fluorescence under ultraviolet (UV) light, is added 20 μL of loading dye is added to the samples and loaded into the gel, along with a DNA ladder as a reference. The gel with the loaded sample was run at 120 volts for 50 minutes.

Table 1. Composition of the PCR reaction samples.

	#1 μL	#2 μL	#3 μL	#4 μL
ddH ₂ O	80	70	78	68
DNA plasmid (10 ng/ μL)	5	5	5	5
Primer forward (100 pmol/ μL)	1	1	1	1
Primer reverse (100 pmol/ μL)	1	1	1	1
Native pfu Buffer (10X)	10	10	10	10
dNTP (10 nM)	2	2	2	2
DMSO	0	10	0	10
MgSO ₄ (100 mM)	0	0	2	2
Native pfu polymerase	1	1	1	1
Total	100	100	100	100

2.2 Gel Extraction with QIAquick Gel Extraction Kit Protocol

The DNA PCR products was cut out from the agarose gel and extracted from the gel through Gel extraction with the QIAquick Gel Extraction Kit Protocol. The DNA products are cut out and transferred into microcentrifuge tubes, then 900 μL of QG buffer was added to each tube and kept at 55°C until the gels dissolved. The solution was transferred into a QIAquick column and centrifuge for 1 minute at 12k rpm. 750 μL of PE washing buffer with EtOH (ethyl alcohol) is added and incubated at room temperature for 5 minutes. After centrifuging for 1 minute at 12k rpm and discarding the flowthrough, the samples were centrifuged again at same speed for 1 additional minute. The QIAquick column is then inserted into a 1.5 mL tube and 30 μL ddH₂O was added in the center of column, then incubated at room temperature 2 minutes. The 1.5 mL

tube was centrifuged at the same speed for 1 minute and the DNA concentration was measured by UV at 260 nm.

2.3 Restriction Digestion for Cutting the Target Gene and Vector

When adding the components which are in Table 2, ddH₂O was added first and enzymes were added last. Restriction enzymes were kept in a -20°C box to maintain their activity. The digestions were incubated at 37°C for 3 hours. After the incubation, the DNA plasmids were purified with the PCR purification kit. From the PCR purification kit, 300 μ L of PBI binding buffer was added to each sample at a 5:1 volume ratio. Then the samples were put in the Q1Aquick spin columns and centrifuged for 1 minute at top speed. 750 μ L of PE buffer containing EtOH was added into the samples and incubated at room temperature for 5 minutes. The samples were then centrifuged for 1 minute at top speed, and the flow-through was discarded after centrifuging again for 1 additional minute. Q1Aquick columns were placed in sterile 1.5 mL microcentrifuge tubes and 30 μ L of ddH₂O was added in the center of each column. After incubating at room temperature for 2 minutes and centrifuged for 1 minute, the flow-through was collected and the concentration was measured. The PCR product and the plasmid vector concentrations were measured by UV at 260 nm.

Table 2. Composition of the digestion reaction of target gene and vector.

	PCR Product PRMT5	Plasmid vector
	μL	μL
DNA	30	10
ddH ₂ O	18	32
Buffer	6	6
BSA 10X	6	6
Restriction Enzyme1	3	3
Restriction Enzyme2	3	3
Total	66	66

2.4 DNA Ligation of Target Gene and Vector

In the experiment, 3 samples are necessary. The ratios of target genes and vectors, both of which have been digested by restriction enzymes, are 1:1, 3:1 and one negative control sample. The negative control contains only the vector and it is used to check different growth situation of bacteria after transformation. Like previously, ddH₂O was added first and T4 DNA ligase was added last and was kept in a -20°C box. The three samples were then reacted at 16°C overnight.

2.5 Transformation for Using Bacteria to Amplify Constructed Plasmids

Three aliquots of 50 μL of XL1-Blue supercompetent cells from the -80°C freezer were put in their own 1.5 mL eppendorf tube and then thawed on ice for 10 minutes. Then 0.85 μL of beta-mercaptoethanol (1.4M) was added to each tube and kept on ice for 10 minutes, followed by 10 μL of DNA ligation solution. The solution was swirled gently to mix and then incubated on

ice for 30 minutes. The tubes were then heated at 42°C for 45 seconds and put on ice for 2 additional minutes. 0.5 mL of pre-heated NZY-Broth (NZY-Broth solution is made up of 1 mL NZY media, 12.5 µL of 1M MgCl₂, 12.5 µL of 1M MgSO₄, 20 µL of 20% glucose) was added to each tube. The tubes were incubated at 37°C for 1 hour with rotation (rotation of 225 rpm). Agar plates were pre-warmed at 37°C for 1 hour. 50 µL of the mixed solution is added to an agar plate. The rest of the solution was centrifuged at 5000 rpm for 1 minute at room temperature. Most of the supernatant was discarded and the remaining solution, which is about 50-100 µL, was gently mixed and applied to another agar plate. Around 7 beads were added on the plates and the plates were then shaken to let the bacteria distribute uniformly on the surface of the agar. Beads were then poured out and the plates (1:1 A and B plates 3:1 A and B plates and Control A and B plates) were put in the incubator at 37°C overnight. The following day, the agar plates were taken out and checked for bacteria colonies. The agar plates were then sealed and stored at 4°C. In the afternoon of the same day, ten (5 from 1:1 and 5 from 3:1) colonies were chosen and incubated in 8 mL of LB media with ampicilin (AMP) or kanamycine (Kana) antibiotics. Then, the tubes with LB media were put into the incubator at 37°C overnight with rotation at 225 rpm. The next day, the bacterial cultures were taken out of the incubator and 20 µL of the LB culture media was kept from each sample as seeds and stored at 4°C. The culture tubes were centrifuged at 3000 rpm for 10 minutes at 4°C to discard supernatant. Cell pellets were resuspended thoroughly with 250 µL of Cell Resuspension Solution. The solution was then transferred to 1.5 mL tubes and 250 µL of Cell Lysis solution was added to each sample and inverted 4 times inverted to mix. 10 µL of Akaline Protease Solution was then added and inverted 4 times to mix. The solution was incubated at room temperature for 5 minutes. Then 350 µL of Neutralization Solution was added and inverted ten times to mix and then centrifuged at top speed for 10 minute at room

temperature. Spin columns were inserted into collection tubes and the cleared lysate was decanted into the Spin Columns and centrifuged at top speed for 1 minute at room temperature. Flowthrough was discarded and columns are reinserted into collection tubes. 750 uL of wash solution (ethanol added) was added in the solution and centrifuged at top speed for 1 minute. Flowthrough was removed and the column was reinserted into collection tubes and 250 uL of wash solution was added into the columns. After centrifuging, the flowthrough was discarded and centrifuged for additional 2 minutes. The Spin columns were transferred to sterile 1.5 mL microcentrifuge tubes and 40 uL of nuclease-free water is added into the spin columns. The tubes were centrifuged at top speed for 1 minute at room temperature and the flowthrough containing the DNA plasmid was collected.

2.6 Expression of His-tagged Protein

First, DNA plasmid was transformed into BL21 (DE3), and two colonies were chosen for LB culture overnight. Then LB media culture with bacteria is poured in two flasks, each with 1 L of LB media with Kana or AMP antibiotics. The culture tubes were then put in the 37°C incubator with shaking at 225 rpm. Two hours later, the O.D. values of the media in the flasks were checked every 10 minutes until the value was in the range of 0.6 to 0.8. The flasks were then taken out and cooled down in the 4°C refrigerator. When the flasks were cooled, 0.3 mL of IPTG (1 M) was added into flasks and shaken at 255 rpm in 16 °C overnight. On the second day, to get the pellets, each 1 L of culture liquid was centrifuged in three 500 mL bottles at 5000 rpm for 10 minutes in 4°C. Then the supernatant was discarded and the pellets were resuspended with 30 mL Ni-lysis-buffer and the resuspended cells were frozen with liquid N₂ and stored at -80°C. One tube of cells was taken for the French-Press in 4°C. The tube was put in the water to thaw

for 10 minutes, and then the sample was put through the French Press twice with sample coming out drop by drop. The cell lysate was centrifuged immediately for 30 minutes at 14000 rpm at 4°C. 20 µL of supernatant was saved for SDS-PAGE later on. A column with 5 mL of Ni-beads is prepared and washed with 50 mL 1X column equilibrium buffer 5 times. The supernatant of PRMT5 after the French-press was loaded onto the beads and shaking for 1 hour. The beads with the enzyme bound were washed with 50 mL of column equilibrium buffer twice. Then the beads need to be washed with 10 mL of washing buffer 10 times. The washing solution was collected in 8 tubes. Afterwards, the beads need to be washed with 8 mL of elution buffer which washed the PRMT5 out of the and the eluted solution is collected into 6 separate collection tubes. Then 20 µL of the beads were taken out to check on a SDS-PAGE gel. A 12% SDS gel is appropriate for PRMT5. The supernatant and pellets from the French-press, beads before binding, 5 washing samples, 6 elution samples and beads after elution are loaded onto the SDS-PAGE gel. For the solution and supernatant samples, 5 µL of 5X loading dye was added into 20 µL of samples and for the beads and pellets, 80 µL of H₂O and 20 µL of 5X loading dye were added. Then every tube was heated at 100°C for 10 minutes and run on the SDS-PAGE at 200 V. When the blue frontline from the dye arrives at the bottom line, the SDS-PAGE was stopped. The gels were transferred to staining solution and shaken overnight. The next morning, the gels are destained for about 5 hours. Meanwhile, all elution buffers needed to be combined together and centrifuged. During the centrifuging, the storage buffer was added into the protein 3 times to change the buffer making the protein stable. When the final solution volume was around 1 mL, the proteins concentration was measured.

Table 3. Components of Bradford protein concentration measurement.

		2 mg/mL BSA	Protein
H ₂ O	800 μ L	796 μ L	796 μ L
Protein	0 μ L	4 μ L	4 μ L
Brad-ford Dye	200 μ L	200 μ L	200 μ L

One tube of H₂O, three tubes of BSA and three tubes of protein was prepared as shown in Table 3 above. The Brad-ford dye was added every 30 seconds between two tubes. Sample H₂O, BSA 1, BSA 2, BSA 3, Protein 1, Protein 2 and Protein 3 needed to be checked by UV at O.D. 595 nm. The final concentration of the protein was [Average Protein (595 nm)/Average BSA (595 nm)]*2 mg/mL.

Ni-Lysis buffer (Na-HEPES 25 mM PH 7.0, NaCl 500 mM, MgSO₄ 1 mM, Glycerol 10%, PMSF (Phenylmethylsulfonyl fluoride) 1mM with prepared fresh)

Column equilibrium buffer (Na-HEPES 25 mM PH 7.0, NaCl 500 mM, Glycerol 10%, Imidazole 30 mM, PMSF 1 mM with prepared fresh)

Column washing buffer (Na-HEPES 25 mM PH 7.0, NaCl 500 mM, Glycerol 10%, Imidazole 70 mM, PMSF 1 mM with prepared fresh)

Elution buffer (Na-HEPES 25 mM PH 7.0, NaCl 500 mM, Glycerol 10%, Imidazole 200 mM, NaCl 500 mM, EDTA 100 mM)

Storage buffer (HEPES 25 mM PH 7.0, NaCl 500 mM, Glycerol 10%, NaCl 500 mM, EDTA 100 mM, DTT 5 mM)

2.7 Radioactive Assay for Activity Test

PRMT5 was always kept on ice. Peptide H4-20 (the first 20-amino acid peptides in histone H4) was prepared for 7 samples from Sample 1 to Sample 7 with an increasing final concentration of 6.25 μM , 12.5 μM , 25 μM , 50 μM , 100 μM , 200 μM , 400 μM as shown in Table 4. A mix solution composed of 13 μL of ^{14}C labeled AdoMet (400 μM), 200 μL of 2XRB (HEPES: 100 mM PH 8.0, NaCl 100 mM, EDTA 2 mM, DTT 2 mM) and 87 μL of H_2O was also prepared. 18 μL of this solution was added into the 7 samples with different concentrations of H4-20 and H_2O every 30 seconds. After 5 minutes of, 6 μL of PRMT5 (2 μM) was added to sample 1 through sample 7 every 30 seconds. All the samples were kept in 30°C and the total reaction time after adding the enzyme was 45 minutes. When the reaction was completed, 20 μL of the solution was taken from every sample and loaded onto P81 paper separately. The remaining solutions were combined together and 20 μL of the mixture was taken and loaded onto three P81 papers. All the P81 papers were dried in the room temperature for 30 minutes and then sample 0-7 were washed by 1L of NaHCO_3 (50 mM PH 9.0) for 12 minutes, three times. The P81 papers loaded with the mixed sample solution do not wash. After washing, all the P81 paper including sample 1-7 and the three mixtures were put in separate 20 mL disposable scintillation vials and dried in the incubator overnight. A duplicate group of the whole experiment reaction was also done. The next day, 10 mL of scintillation liquid was added into the vials and covered with lids. Then the vials were then put into the scintillation machine to measure the CPM value (37). The general procedure and time is illustrated in Figure 5.

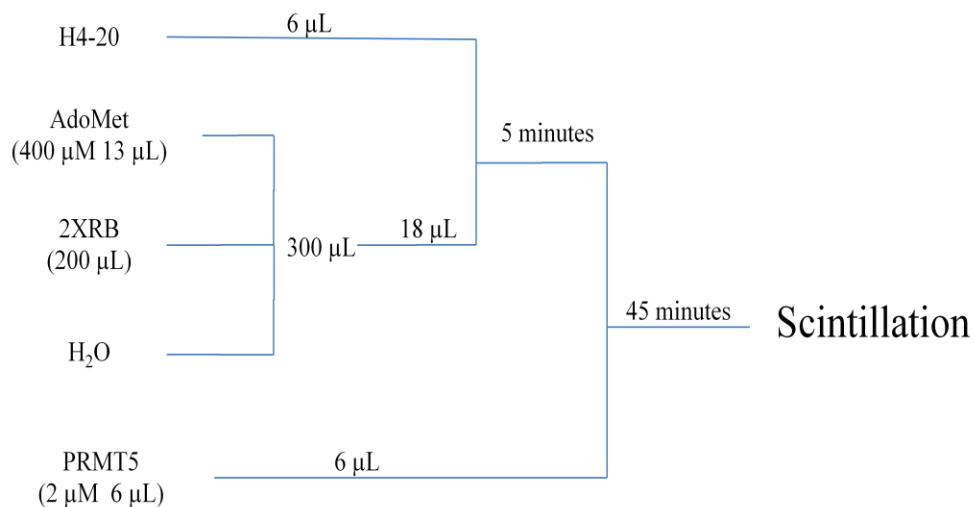


Figure 5. Process and composition of radioactive assay.

Table 4. Composition of the radioactive assay in different substrate concentrations.

Peptide H4-20	0	1	2	3	4	5	6	7
Final (μM)	0	6.25	12.5	25	50	100	200	400
Stock (μM)	H ₂ O	31.2	62.5	125	250	500	1000	2000

2.8 General Radioactive Assay Test

In some cases, proving whether the PRMT5 has activity on H4-20 and H3-20 (the first 20-amino-acid peptides in histone H3) is a priority in addition to figuring out the specific enzymatic properties. Then a consistent concentration of substrate can be applied, with the composition of the assay shown in Table 5.

Table 5. Composition of the general radioactive assay

	H ₂ O	2XRB	Peptide	AdoMet	Protein	Total
H4-20	0 μ L	20 μ L	7 μ L	3 μ L	10 μ L	40 μ L
H3-20	5.6 μ L	20 μ L	1.4 μ L	3 μ L	10 μ L	40 μ L
Control	7 μ L	20 μ L	—	3 μ L	10 μ L	40 μ L

Reaction time was 1-24 hours. The final concentration of H4-20 and H3-20 was 350 μ M. AdoMet concentration was 30 μ M. Protein concentration was flexible. 30 μ L of the total solution was loaded onto P81 papers. After washing for 30 minutes and completely dried, the P81 papers were sent for scintillation. This general test of radioactive assay was straightforward and convenient for figuring out if the proteins have activity to the substrates. This simplified method focuses on the preliminary study of a protein to a substrate.

2.9 Quickchange to Make Truncated PRMT5 Gene/Protein

For the quickchange PCR, the components are shown in Table 6. The PCR program is shown in Table 7. After the PCR, 10 μ L of the product was checked for amplification efficiency on a 1% agarose gel. If a single band was present in the right position, this indicated the efficiency was good. 1 μ L of Dpn I was added to the remaining 40 μ L of PCR product with gentle mixing and then centrifuged for 1 minute at 5000 rpm. The solution is then incubated in 37°C for 1 hour and 1 μ L of the solution was used for transformation into XL1-Blue supercompetent cells for amplification. Three colonies were picked and the DNA is extracted to check for the right DNA sequence. Then the right DNA was used to express the protein.

Table 6. Quickchange PCR reaction system.

	#1	#2	#3
	uL	uL	uL
ddH ₂ O	36	35	32
DNA Template (20 ng/μL)	1	2	5
Primer forward (40 ng/μL)	3	3	3
Primer reverse (40 ng/μL)	3	3	3
Native plus Buffer (10X)	5	5	5
dNTP (10 mM)	1	1	1
Pfu native polymerase	1	1	1
Total	50	50	50

Table 7. Quickchange PCR program.

	T(°C)	Time
1	95	10 min
2	95	30 s
3	55	1 min
4	68	16 min (2 min/kb)
5	2-5	17 times
6	4	forever
7	End	

Chapter 3 Subcloning of PRMT5 Gene into an Expression Vector

3.1 Subcloning PRMT5 into pGEX4T1

Subcloning is a widely used technique which can transfer a gene of interest into a target vector for further studies. pGEX4T1 is a vector with a GST tag that can be used to purify the PRMT5 protein. GST (Glutathione S-transferase) is an enzyme present in both eukaryotes and prokaryotes. GST can be used in genetic engineering to rebuild the plasmid with a GST sequence. Thus, Glutathione-Sepharose 4B beads can be used to bind the GST tag and then the protein will be eluted by elution buffer to obtain a purified product.

3.1a PCR Experiments

PCR (Polymerase Chain Reaction) is an important method in modern biology and biochemistry research which can amplify small amounts of DNA to millions of copies (38). With two well designed primers, which bind to opposite ends of the DNA template, Native pfu DNA polymerase, dNTP, DMSO, MgSO₄ and PCR buffer, a target piece of DNA can be amplified in vitro. Primers were designed around 12-16 nucleic acids which were complementary to each 3' strand of DNA template.

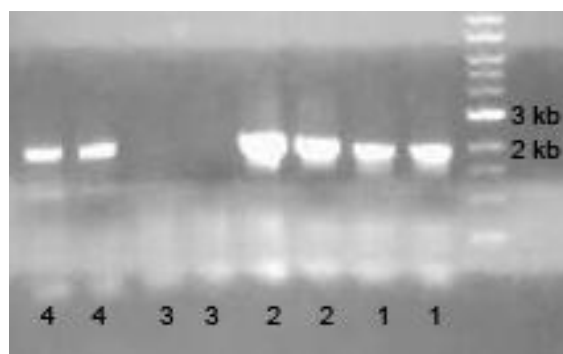


Figure 6. PCR products of PRMT5 DNA. Correct bands are around 1.9 kb. Lanes 1, 2, 3, and 4 are sample 1, 2, 3 and 4. Sample 1 and 2 contain no $MgSO_4$. Sample 1 and 3 contain no DMSO.

Figure 6 shows that samples 1, 2 and 4 have the desired PCR product size, which is around 1.9 kb. The bands were cut and the DNA was extracted out of the gel using Q1Aquick Gel Extraction Kit Protocol and the concentration was measured. (Concentration=O.D. $260 \times 0.05 \times$ dilution factor) Sample 1 and Sample 2 concentrations were 0.0700 mg/mL; sample 3 concentration was 0.0200 mg/mL.

3.1b Restriction Digestion of PRMT5 and pGEX4T1

The target gene PRMT5 and vector pGEX4T1 needed to be constructed into one plasmid, which is a circular double stranded DNA. Plasmid DNA can be transformed into bacteria to express the target gene. The target gene and vector can be cleaved at the same restriction sites, which are specific nucleotide sequence, usually around 6 bases, to make them combine together forming a plasmid. This process is called restriction digestion. Restriction sites have to exist in both the target gene, which are the PRMT5 PCR products and the vector pGEX4T1. These restriction sites can be cleaved by their corresponding restriction enzyme. So finding two restriction sites which exist in both the target gene and the vector is very critical for the

restriction digestion step. The two sites in the gene and the vector can be combined together by DNA ligase. For PRMT5 and pGEX4T1, EcoRI and XhoI were appropriate. Therefore the restriction enzymes EcoRI and XhoI were used to cleave the PRMT5 gene and the pGEX4T1 vector.

PRMT5 was a PCR products, so in the PRMT5 batch, the components were 30 μL of PRMT5, 18 μL of ddH₂O, 6 μL of Buffer4, 6 μL of BSA10X, 3 μL of EcoRI and 3 μL XhoI. In pGEX4T1 batch the components were 10 μL of pGEX4T1, 32 μL of ddH₂O, 6 μL of Buffer4, 6 μL of BSA10X, 3 μL of EcoRI, 3 μL of XhoI. The total volume of each batch is 66 μL . The digestions were incubated in 37°C for three hours. PRMT5 and pGEX4T1 were purified using the PCR purification kit. The concentration of PRMT5 was 0.1225 mg/mL. The molecular weight was 1.2×10^6 Da and molar concentration was 1.0×10^{-7} . pGEX4T1 concentration was 0.1300 mg/mL. Molecular weight equaled to 3.2×10^6 Da and Molar concentration was $0.1300/3.2 \times 10^6 = 0.4 \times 10^{-7}$ M.

3.1c DNA Ligation to Rebuild the Plasmids

The target gene and vector after the digestion have sticky ends of DNA. So to combine these two as a plasmid, it requires the enzyme DNA ligase to reconstruct the hydrogen bond at the restriction sites. The function of DNA ligase is to recreate covalent phosphodiester bonds between 3' ends and 5' ends of two single stranded DNA, which were cut by the restriction enzymes. In this reaction, ATP is required to provide energy and DNA ligase buffer is also needed. In this step, 3 groups of samples with different ratios of target gene and vector were setup. 1:1 group included 1 μL of PRMT5, 3 μL of pGEX4T1, 21.5 μL of ddH₂O, 3 μL of T4buffer, 1.5 μL of T4DNA ligase. 3:1 group includes 3 μL of PRMT5, 3 μL of pGEX4T1, 19.5

μL of ddH₂O, 3 μL of T4buffer, 1.5 μL of T4DNA ligase. Negative control group includes 0 μL of PRMT5, 3 μL of pGEX4T1, 22.5 μL of ddH₂O, 3 μL of T4buffer, 1.5 μL of T4DNA ligase. Every group had a total volume of 30 μL . The ligation reaction was carried out in the 16°C incubator overnight.

3.1d Transformation of the Recombinant Plasmid into XL1 Blue Bacteria

Some bacteria like *E. coli* have the ability of taking up foreign genes. pGEX4T1-PRMT5 plasmid after ligation was transformed into XL1 Blue, which can replicate the DNA efficiently. The batches of 1:1, 3:1 and a negative control were transformed in to XL1-Blue and cultured on agar plates. The next day, 5 colonies from 1:1 and 5 colonies from 3:1 were picked and cultured in 8 mL LB media overnight. The plasmid from these colonies were extracted and digested with EcoRI and XhoI for 3 hours again. However, the volume was changed to 20 μL , which includes 10 μL of DNA, 4 μL of ddH₂O, 2 μL of Buffer4, 2 μL of BSA10X, 1 μL of EcoRI and 1 μL of XhoI. Then the DNA after digestion was run on a 1% agarose gel to check the efficiency of ligation which was a critical step to figure out whether the subcloning was successful.

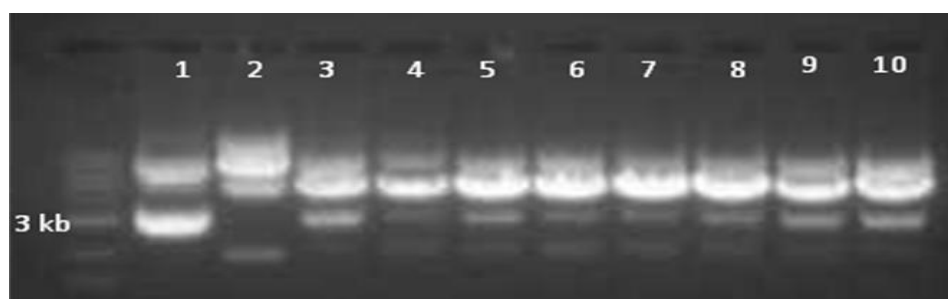


Figure 7. Checking the ligation efficiency of PRMT5 efficiency with restriction digestions. Lanes 1-10 are ten representative samples.

From the results of the gel image of Figure 7, samples 2 and 6 were in the right position

compared to the marker of 1 kb. So DNA samples 2 and 6 which were extracted from the colonies were sent for sequencing to verify the identity of the PRMT5 in the plasmid and the original PRMT5 sequence. In the BLAST tool of NCBI which is shown in Figure 8, 97% identities showed the two PRMT5 sequences were almost the same. This indicates that the subcloning of PRMT5 to pGEX4T1 is successful.

```

Score = 2063 bits (1073), Expect = 0.0
Identities = 1197/1229 (97%), Gaps = 15/1229 (1%)
Strand=Plus/Plus

Query 54 ATGGCGGCGATGGCGGTGCGGGGTGCTGGTGGGAGCCGCGTGTCCAGCGGGAGGGACCTG 113
      |||
Sbjct 1 ATGGCGGCGATGGCGGTGCGGGGTGCTGGTGGGAGCCGCGTGTCCAGCGGGAGGGACCTG 60

Query 114 AATTGCGTCCCCGAAATAGCTGACACACTAGGGGCTGTGGCCAAGCAGGGGTTTGATTC 173
      |||
Sbjct 61 AATTGCGTCCCCGAAATAGCTGACACACTAGGGGCTGTGGCCAAGCAGGGGTTTGATTC 120

Query 174 CTCTGCATGCCTGTCTTCCATCCGCGTTTCAAGAGGGAGTTTCATTAGGAACCTGCTAAG 233
      |||

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Figure 8. Alignment of two sequences for checking the identity.

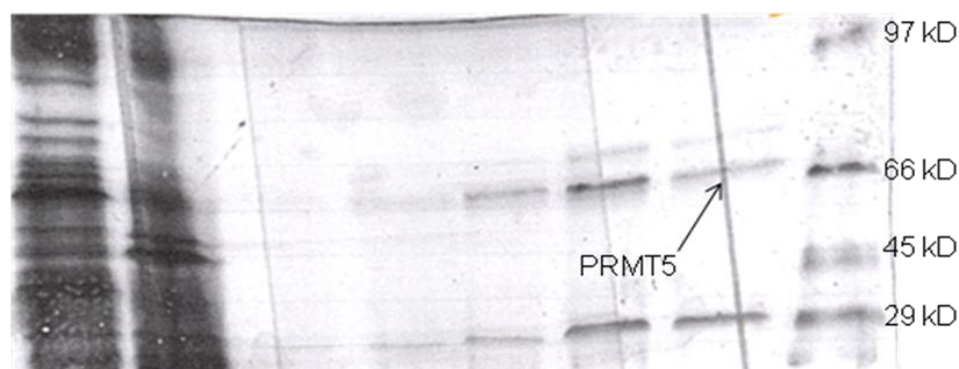


Figure 9. SDS-PAGE of expressed of GST-tagged PRMT5.

The gel image shown in Figure 9 showed that pGEX4T1-PRMT5 was expressed in BL21 (DE3). The PRMT5 made for the radioactive assay was prepared by another student Arpana Sagwal. The result showed that there was no activity on H4 and H3.

3.2 Subcloning of shPRMT5 into pTXB1 and pET28a

3.2a Preparation of shPRMT5 from puc57-shPRMT5 Plasmid

Synthetic human PRMT5 (shPRMT5) was obtained from the GenScript company, which has changed the nucleotide sequence of PRMT5 through gene codon optimization analysis to optimize the PRMT5 gene via removing inhibition sites, repeated sequence, restriction sites which may interfere with the cloning, and negative CpG islands. The PRMT5 gene can be expressed well in E coli. without modifying the amino acid sequence. The shPRMT5 gene was originally isolated from the puc57 vector. The concentration of the plasmid puc57-shPRMT5 was 0.4675 mg/mL; the concentration of pTXB1 was 0.1175 mg/mL and the concentration of pET28a was 0.1375 mg/mL. The DNA samples were digested by restriction enzymes NdeI and XhoI. The components are 6 μ L of buffer4, 3 μ L of NdeI and 3 μ L of XhoI, 6 μ L of BSA10X in every group. pTXB1 was 10 μ L and ddH₂O was 32 μ L; pET28a was 10 μ L and ddH₂O was 32 μ L; puc57-shPRMT5 was 20 μ L and ddH₂O was 22 μ L. Each digestion had a total volume of 60 μ L. The digestion was incubated in 37°C for three hours. Then the puc57-shPRMT5 after digestion was separated via agarose gel electrophoresis. In Figure 10, five major bands appeared on the gel. The lowest bands (band E) were shPRMT5 and they were cut out to purify the DNA.

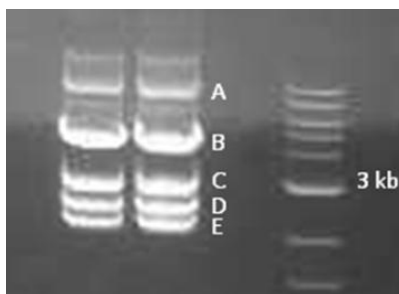


Figure 10. Band A is unclear. Band B are puc57-shPRMT5 plasmid. Band C is puc57 vectors. Band D is unclear. Band E is shPRMT5.

3.2b Insertion of shPRMT5 into pTXB1 and pET28a

The concentration of shPRMT5 from the lowest band was 0.0475 mg/mL. Then pET28a and pTXB1 after digestion with NdeI and XhoI restriction enzymes were purified by PCR purification kit. Concentration of pTXB1 was 0.025 mg/mL and concentration of pET28a was also 0.025 mg/mL. Table 8 showed the molar ratio of shPRMT5, pTXB1 and pET28a. And in Table 9, according to Table 8 results, ligation reaction system was set up.

Table 8. Calculations of the concentration and molecular weight (MW) of PRMT5, pTXB1 and pET28a.

	Concentration (mg/mL)	Base Pair	MW (Da)	M (pmol/ μ L)
shPRMT5	0.0475	1900	1235000	0.038
pTXB1	0.025	6700	4355000	0.0057
pET28a	0.025	5371	3491150	0.0072

Table 9. Setup of the ligation reaction.

	pTXB1		pET28a	
	Molar ratio of target gene over vector		Molar ratio of target gene over vector	
	3:1	6:1	3:1	6:1
shPRMT5	3.8 μ L	7.6 μ L	3.8 μ L	7.6 μ L
vector	8.4 μ L	8.4 μ L	6.7 μ L	6.7 μ L
ddH ₂ O	13.3 μ L	9.5 μ L	15 μ L	11.2 μ L
T4Buffer	3 μ L	3 μ L	3 μ L	3 μ L
T4Ligase	1.5 μ L	1.5 μ L	1.5 μ L	1.5 μ L
Total	30 μ L	30 μ L	30 μ L	30 μ L

Each ligation reaction had 10 μ L taken for transformation into XL1-Blue and cultured on agar plates. 17 colonies of pTXB1-shPRMT5 and 20 colonies of pET28a-shPRMT5 were picked out and after culturing each of them in 8 mL LB media, the plasmid DNA was extracted. Then 10 μ L of DNA from the colonies was taken for digestion with NdeI and XhoI enzymes again. The digested DNA was then run on a 1% agarose gel to check the result. The agarose gel in Figure 11 indicates the results of pTXB1-shPRMT5 ligation and the agarose gel in Figure 12 indicates the results of pET28a-shPRMT5 ligation.\



Figure 11. DNA bands on a 1% agarose gel for checking the ligation efficiency of shPRMT5 and pTXB1. Lanes 1-17 are representations of DNA plasmids after restriction enzyme cutting of bacteria colonies 1-17

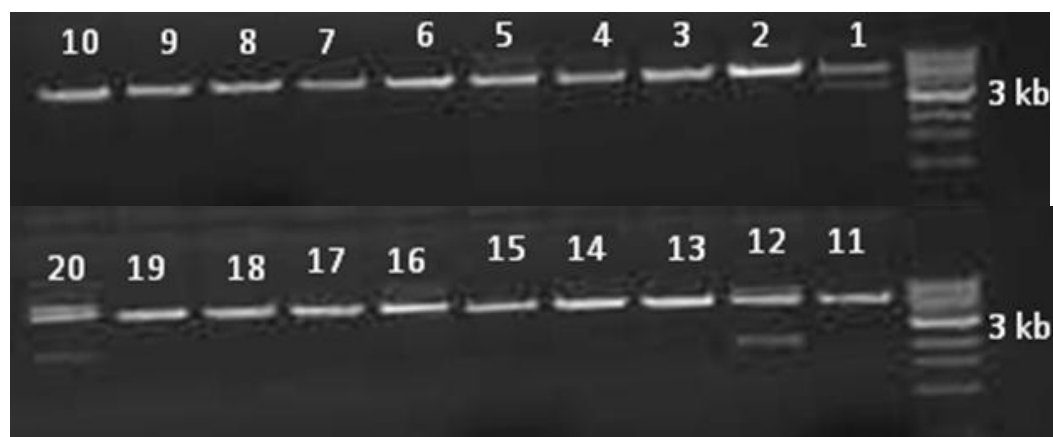


Figure 12. DNA bands on a 1% agarose gel for checking the ligation efficiency of shPRMT5 and pET28a. Lanes 1-20 are representations of DNA plasmids after restriction enzyme cutting of bacteria colonies 1-20.

In the pTXB1-shPRMT5 gel, there was no band in the correct position, which is around 1.9 kb. In pET28a-shPRMT5, however, lane 12 and 20 contained bands in the 1.9 kb position which

is the length of shPRMT5. So the primers T7 and T7 terminator were used to sequence sample 12. The result showed that shPRMT5 sequence is the same as shPRMT5 in puc57 vector, and pET28a had almost the same sequence.

3.3 Subcloning shPRMT5 into pTXB1

To get more linear shPRMT5, puc57-shPRMT5 was taken for PCR with a cloned DNA polymerase and the modification of the temperature in the second step to 62°C. shPRMT5 products were checked on a 1% agarose gel and found a band in the correct position, which was around 1.9 kb. Since the PCR reaction was successful, DNA was extracted from the gel and the concentration was measured, which was 0.15 mg/mL. The PCR products were digested by NdeI and XhoI enzymes, along with shPRMT5-pET28a which was also digested by the same enzymes to act as a control to confirm shPRMT5 is in the right position on the agarose gel. The picture of the agarose gel showed that the digestion of the shPRMT5 PCR product was successful, and the digested shPRMT5 concentration was 0.065 mg/mL. A ligation reaction was set up at a molar ratio of 6:1 (shPRMT5:pTXB1). After transformation into XL1-Blue and cell lysis, plasmids of pTXB1-shPRMT5 were digested again with NdeI and XhoI enzyme to check the ligation efficiency. However, there was no successful constructed plasmid shown on the 1% agarose gel image. In order to make sure that the vector pTXB1 was digested completely, pTXB1 bands were cut out of the gel and extracted. Then pTXB1 was digested again and after purification, the concentration of pTXB1 was 0.05 mg/mL. shPRMT5 and pTXB1 were used to set up two ligations again with molar ratios of 6:1 and 3:1. After transformation into XL1-Blue and cell lysis, the plasmids were digested with NdeI and XhoI and checked on an agarose gel. From thirty colonies, some successful plasmid constructs were shown in the gel picture of Figure 13. Sample

6 and sample 13 were sent for sequencing and the results indicated that the plasmids were both the same as pTXB1-shPRMT5. Concentration of sample 6 was 0.105 mg/mL and concentration of sample 13 was 0.095 mg/mL.

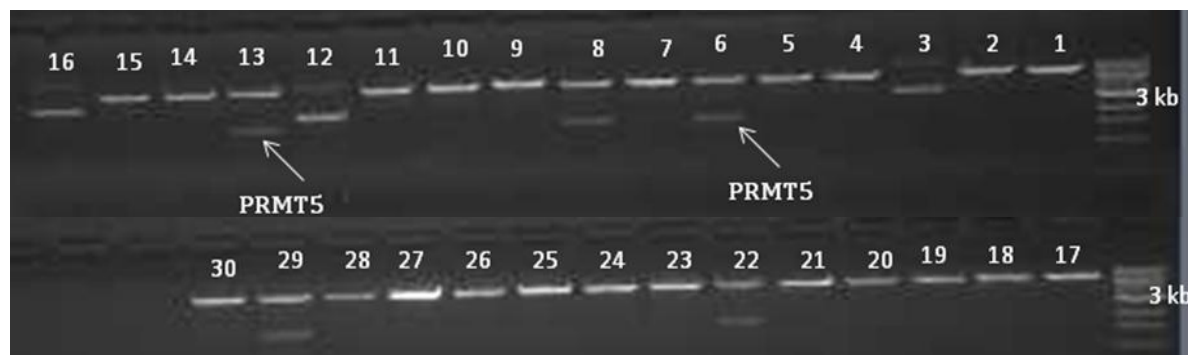


Figure 13. DNA bands on a 1% agarose gel for checking the ligation efficiency of the second sub-cloning attempt of shPRMT5 and pTXB1. Lanes 1-30 representation of DNA plasmids after restriction enzyme cutting of bacteria colonies 1-30.

Chapter 4 Expression of His-Tagged shPRMT5 Protein

pET28a is a widely used vector in biochemistry, which contains restriction sites such as NdeI, XhoI, EcoRI and a 6X-His tag sequence (39). In certain E. coli strains, it can be expressed at a high efficiency, like for instance in BL21 (DE3). The selectable marker of pET28a is Kanamycin. This pET28a vector contains a 6X His-tag sequence and after its expression, the 6 histidines will be fused to the shPRMT5 products. The fused 6X His-tag will then be purified by a nickel affinity column. The histidine structure contains an imidazole ring. Both imidazole and histidine can bind to Ni-beads. Therefore, competition can be used to purify the His-tagged protein. At low concentrations, imidazole can bind to nickel ions that prevent nonspecific and low-affinity binding to the nickel column, which then can be washed out, and the 6X His-tagged protein will remain bound to the Ni-beads. However, at a high concentration of imidazole, the 6X His-tagged proteins will eluted out since there is higher competition for the Ni-beads. IPTG is required for expression of the protein of interest in the BL21(DE3) cells at 16°C. IPTG is an analog of the metabolite lactose, which can trigger the transcription of the lac operon. Additionally, IPTG can induce transcription constitutively, since it can not be metabolized in BL21 (DE3) cells. After lysing the cells using the French-press twice and centrifuged, the supernatant was purified with Ni-beads and washed by 50 mL equilibrium buffer twice. Then it was washed by 10mL washing buffer for 10 times and 8 mL elution buffer for 6 times. The supernatant, pellets, washing solution and elution solution were checked on a 12% SDS-PAGE. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is widely used in biochemistry and molecular biology to check the protein fraction by molar weight through their electrophoretic mobility (40). Sodium dodecyl sulfate (SDS) can separate the proteins by binding to amino acid residues to give a uniform negative charge on the protein and dissolve hydrophobic

molecules. Consequently, proteins lose their secondary, tertiary and quaternary structures which leave a linear structure binding to SDS with negative charge. Acrylamide can become polyacrylamide spontaneously when dissolved in the water and form different sized pores. During electrophoresis, electric current can induce the negative charged protein molecules to move through the polyacrylamide gel which is cross-linked to make the protein molecules move solely on their sizes. The small sized molecules run faster, while the larger sized molecules run slower. After staining the protein, the band will show that the different sized bands of protein molecules which can be compared to a standard marker.

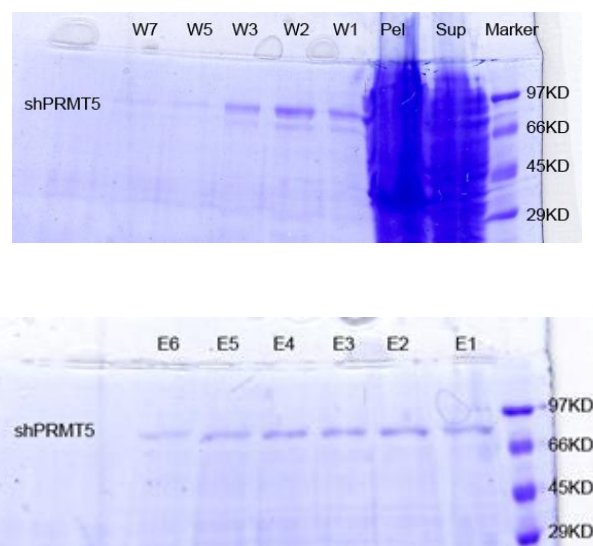


Figure 14. 12% SDS-PAGE with supernatant, pellets, washing solution 1, 2, 3, 5, 7 samples in upper gel. Elution solution 1, 2, 3, 4, 5, 6 samples in lower gel. PRMT5 protein at 70 kD position.

The SDS gel in Figure 14 shows that the correct shPRMT5 bands are at the 70 kD position. The protein was then condensed to get a higher concentration and changed with 135 mL of storage buffer with DTT. The concentration of shPRMT5 was 0.164 mg/mL Furthermore, to

make the radioactivity assay more efficient, the concentration of protein needed to be higher. So shPRMT5 was centrifuged from 1.2 mL to 200 uL and 60 uL of shPRMT5 was taken and 15 uL of dye was added to run on a 12% SDS-PAGE. The concentration of shPRMT5 came out to be 0.2824 mg/mL.

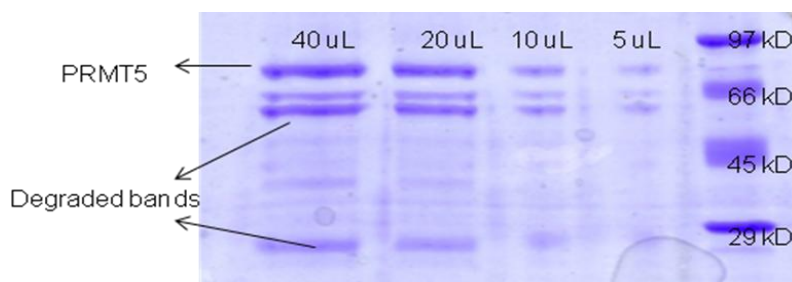


Figure 15. 12% SDS-PAGE with shPRMT5 protein. It seems that PRMT5 has degraded into two major bands at around 60 kD and 25 kD.

Based on Figure 15, the sum of two degraded bands molecular weights equals to molecular weight of shPRM5. The bands are clear in different volumes loaded on the gel. So it is possible that some shPRMT5 had degraded due to unknown reasons. The next step is to check the enzymatic activity with a radioactive assay.

Chapter 5 Enzymatic Activity of shPRMT5

5.1 Radioactive Assay of His-tagged shPRMT5

To investigate if the shPRMT5 had activity on H4-20 and H3-20, a general test of activity of shPRMT5 was performed with the composition of Table 10. A mixture of 2XRB, H4-20, H3-20, AdoMet and enzyme with Peptide (H4 and H3) final concentration of 350 μ M and shPRMT5 final concentration of 1 μ M was prepared.

Table 10. General test of shPRMT5 activity on H4-20 and H3-20.

		H ₂ O	2XRB	Peptide	AdoMet	PRMT5	Total
shPRMT5	H4-20	0 μ L	20 μ L	7 μ L	3 μ L	10 μ L	40 μ L
	H3-20	5.6 μ L	20 μ L	1.4 μ L	3 μ L	10 μ L	40 μ L
	Control	7 μ L	20 μ L	-	3 μ L	10 μ L	40 μ L

The CPM values of shPRMT5 were 3590 for H4, 310 for H3 and 112 for the control which was background. The results are shown in Table 11. There was activity of shPRMT5 on H4-20 which was obviously higher than on H3-20.

Table 11. Results of activity assay of shPRMT5 on H4-20 and H3-20.

	CPM
H4-20	3509
H3-20	301
Negative control	112
Mixture	74002

The general radioactivity assay of shPRMT5 needed to be repeated with the same procedures. The CPM values were 2073 for H4, 270 for H3 and 126 for background and mixture

value is 80986 in Table 12, which show that the activity decreases a little bit but still has activity of shPRMT5 on H4-20 and H3-20.

Table 12. Results of duplicate activity assay of shPRMT5 on H4-20 and H3-20.

	CPM
H4-20	2073
H3-20	270
Negative control	126
Mixture	80986

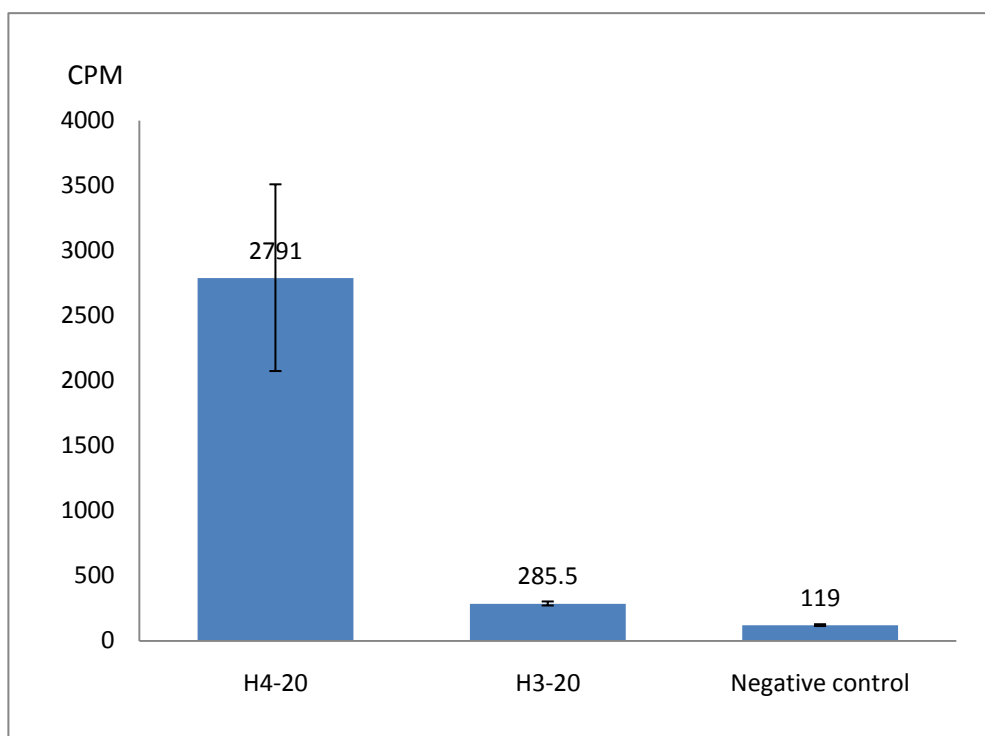


Figure 16. Radioactivity results for shPRMT5 to H4-20 and H3-20.

The results of the reaction shown in Figure 16 indicate that the results are repeatable and reasonable. PRMT5 has a higher activity on the H4-20 substrate and lower activity on the H3-20

substrate *in vitro*. This is an important result and it supports the need to conduct future experiment to study the enzymatic relation between PRMT5 and H4-20.

5.2 Degradation of shPRMT5

As the previous data showed, there was activity of shPRMT5 on H4-20 and H3-20. However, through the SDS-PAGE gel in Figure 17, multiple bands were found, which showed that shPRMT5 degraded further more. To collect the further degraded shPRMT5 protein, the washing solution was combined and condensed with centrifugation and changed in the storage buffer with DTT. The condensed solution concentration of the protein was 1.15 mg/mL.

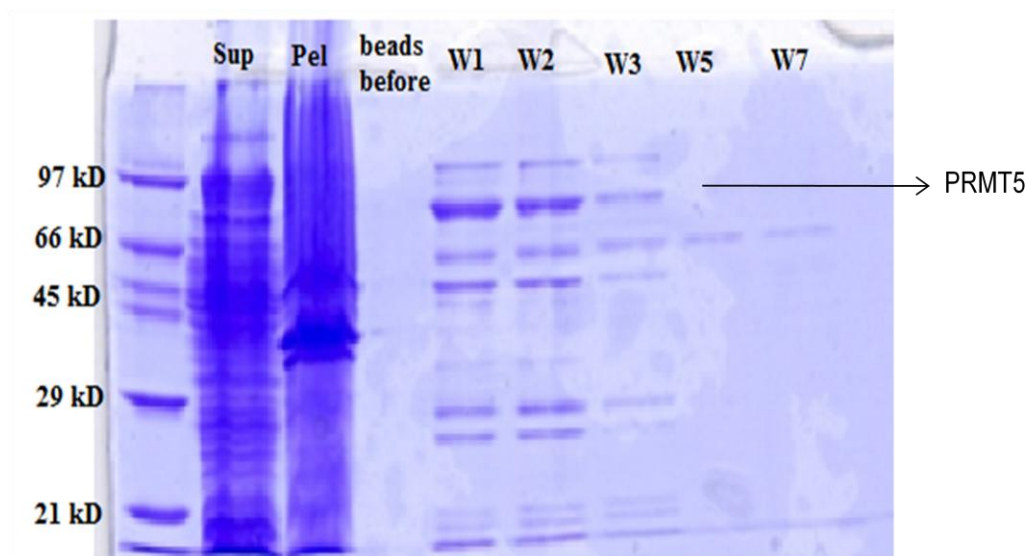


Figure 17. 12% SDS-PAGE Gel representation of the shPRMT5. shPRMT5 degraded further and generated multiple bands.

5.3 Radioactive Assay of Degraded shPRMT5

His-tagged shPRMT5 protein was expressed once more with the same steps as the previous time. However, this time, most proteins were washed out by washing solution and almost none

was in the elution solution whose concentration of imidazole was 200 mM. So the washing solution was collected and condensed with centrifugation and run on a SDS-PAGE. There were multiple bands present in the gel pictures. To figure out whether these proteins had activity on H4-20 and H3-20, a general radioactive assay test was set up with the composition of Table 13. The total proteins concentration was 1.15 mg/mL and the reaction time was 1 hour.

Table 13. Composition of additional general radioactive assay of shPRMT5 on H4-20 and H3-20.

	H ₂ O	2XRB	Peptide	AdoMet	shPRMT5	Total
H4-20	0 μ L	20 μ L	7 μ L (2 mM)	3 μ L (400 μ M)	10 μ L (16 μ M)	40 μ L
H3-20	5.6 μ L	20 μ L	1.4 μ L (10 mM)	3 μ L (400 μ M)	10 μ L (16 μ M)	40 μ L
Negative Control	7 μ L	20 μ L	—	3 μ L (400 μ M)	10 μ L (16 μ M)	40 μ L

Table 14. Results of additional radioactive assay of shPRMT5 on H4-20 and H3-20.

	CPM
H4-20	248
H3-20	180
Negative Control	131
Mixture	71878

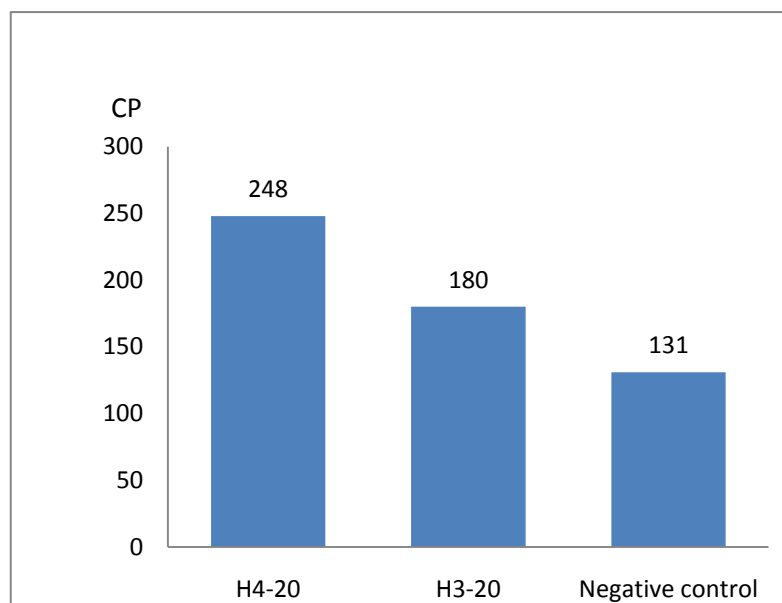


Figure 18. Radioactivity results for shPRMT5 on H4-20 and H3-20.

Based on the results of the scintillation in Table 14 and Figure 18, activity of shPRMT5 on H4-20 and H3-20 was present. However, the activity of shPRMT5 on H4-20 was higher and lower on H3-20. Compared to the first time expression of shPRMT5, the activity on H4-20 and H3-20 decreased dramatically. Therefore, fragments of the whole shPRMT5 protein may still have activity. To investigate which part of protein contains the active site, the proteins should be separated and another radioactivity test would be a logical research plan in the future. In addition, to analyze it together with the protein expressed the first time, SDS-PAGE would be helpful and reasonable. FPLC (Fast protein liquid chromatography) can be applied as a feasible method to separate proteins with different molecular weights with these setting shown in Figure 19. In FPLC, the size exclusion chromatography column was used.

5.4 FPLC of shPRMT5.

Method Information		Start Protocol		Questions		Result Name	
Variables	Scouting	Notes	Gradient	BufferPrep	Columns	Reference Curves	Evaluation Procedures
Block	Variable	Value	Range				
Main	Column (ml)	180.000	0.100 - 999999.000				
Start_with_PumpWash_Basic	D Wash_Inlet_A	Off					
	D Wash_Inlet_B	Off					
Flow_Rate	Flow_Rate (ml/min)	1.000	0.000 - 10.000				
Column_Pressure_Limit	Column_PressureLimit (MPa)	1.20	0.00 - 25.00				
Start_Instructions	D Averaging_Time_UV	5.10					
Start_Conc_B	D Start_ConcB (%B)	0.0	0.0 - 100.0				
Column_Equilibration	D Equilibrate_with (CV)	0.00	0.00 - 999999.00				
Aut_PressureFlow_Regulation	D System_Pump	PressFlowControl					
	D System_PressLevel (MPa)	1.15	0.00 - 25.00				
	D System_MinFlow (ml/min)	0.500	0.000 - 10.000				
Sample_Injection	D Empty_loop_with (ml)	1.00	0.00 - 999999.00				
Fractionation	Eluate_Frac_Size (ml)	2.000	0.000 - 50.000				
	Peak_Frac_Size (ml)	0.000	0.000 - 50.000				
Length_of_Elution	Length_of_Elution (CV)	1.50	0.00 - 999999.00				

Show details
 Show unused variables
 Display tooltip for extended variable cells

[Edit Variable...](#) [Help](#)

Figure 19. FPLC settings.

Tube 33-35 (No. 1), 36-39 (No.2), 39-41(No.3), 45-48(No.4) and 52-57(No.5) were chosen for collection and after condensing, concentrations were measured and checked via SDS-PAGE gel of Figure 20. Concentration of No.1 was 0.52 mg/mL; No.2 was 0.75 mg/mL; No.3 was 0.27 mg/mL; No.4 was 0.18 mg/mL and No.5 was 0.38 mg/mL

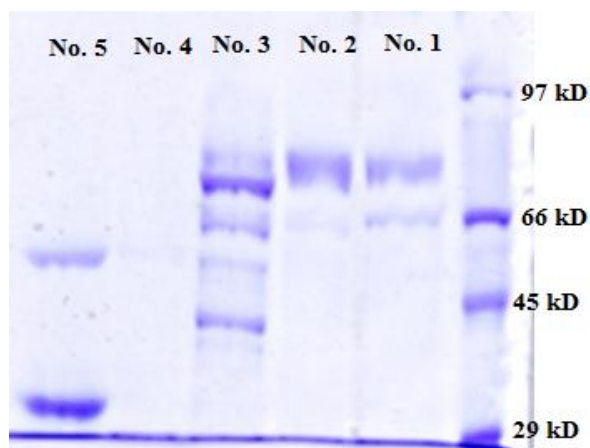


Figure 20. 12% SDS-PAGE gel with different groups of proteins with different molecular weights separated by FPLC.

5.5 Radioactive Assay of Different Protein from shPRMT5 on H4-20.

Every protein fraction from FPLC was tested with the radioactive assay for enzymatic activity. The composition is shown in Table 15. Reaction time was 1 hour. Final concentration of H4-20 was 350 μM and SAM was 30 μM . Then the samples were checked by liquid scintillation to obtain the CPM values.

Table 15. Composition of radioactive assay of different sections of shPRMT5 on H4-20.

	H ₂ O	Peptide H4-20 (2 mM)	Protein	SAM (400 μM)	Total
Control	37 μL	-	-	3 μL	40 μL
No. 1	-	7 μL	30 μL	3 μL	40 μL
No. 2	-	7 μL	30 μL	3 μL	40 μL
No. 3	-	7 μL	30 μL	3 μL	40 μL
No. 4	-	7 μL	30 μL	3 μL	40 μL
No. 5	-	7 μL	30 μL	3 μL	40 μL
Original protein	-	7 μL	30 μL	3 μL	40 μL

Table 16. Results of the five sections of shPRMT5 activity on H4-20 methylation.

	CPM
Control	91
No. 1	88
No. 2	96
No. 3	103
No. 4	81
No. 5	81
Original protein	191

The results in Table 16 showed that the five sections of the separated proteins CPM values are quite low and close to the control values. It is possible that these 5 groups have no activity. In order to confirm this, the reaction time was elongated to 20.5 hours and the same reaction was made again with the same components.

Table 17. Result of general test of radioactive assay of 5 sections after modifying the reaction time to 20.5 hours.

	CPM
Control	189
No. 1	334
No. 2	444
No. 3	452
No. 4	219
No. 5	359
Original protein	712

However, certain sections had an increased in CPM values showed in Table 17 and this verifies that some of these protein fragments have activity on H4-20. To make the data more clear, every group CPM value was subtracted by the control CPM value to get the modified data. These data were divided by No.1, No.2, No.3, No.4 and No.5 concentrations separately to get the normalized values as in Table 18 and Figure 21.

Table 18. Results of the activity of 5 group normalized concentrations on H4-20.

Protein group	Minus control CPM	Divide by concentration
No.1	145	278.9
No.2	225	340
No.3	263	974
No.4	30	166
No.5	170	447
Original protein	523	454

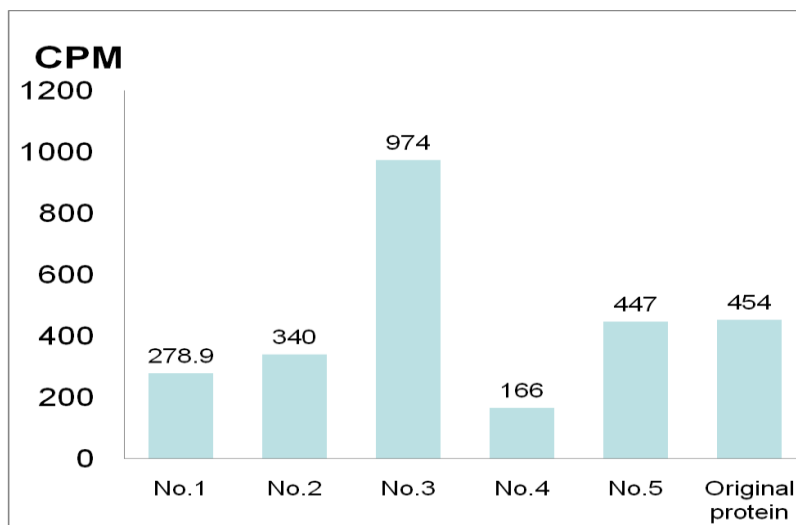


Figure 21. Activity of 5 fractions of the shPRMT5 and original shPRMT5.

To obtain clearer bands of No.1, No.2, No.3 No.4 and No.5 proteins on the SDS-PAGE gel, the volume of each group was increased to 40 μ L and the original protein to 20 μ L, 40 μ L of the previous shPRMT5 was also loaded as a control.

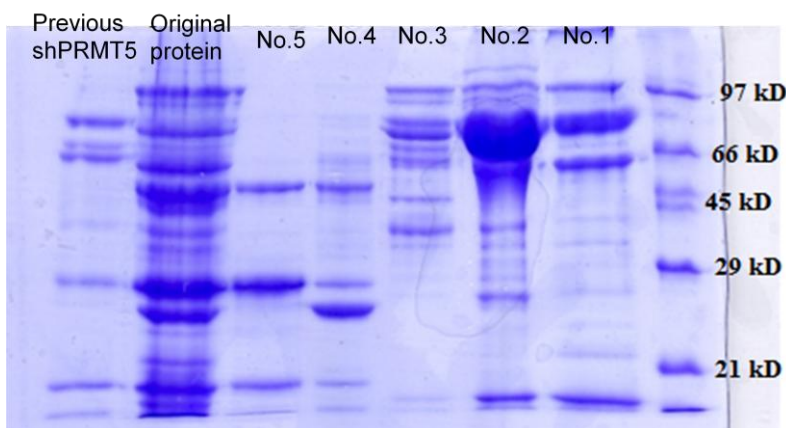


Figure 22. 12% SDS-PAGE gel showing the samples after condensation of the different groups of protein and the previous shPMRT5 as the control.

The SDS-PAGE gels of Figure 22 indicated that the complete shPRMT5 had certain

activity on H4-20. However, No.3 had a protein fragment around 66 kD, which might contain the active site, since it has a high CPM value. Furthermore, No.5 has high activity and a band around 48 kD, which also is presented in the degraded shPRMT5. Therefore, if shPRMT5 was cut into different sections, it is possible to identify the active region of shPRMT5.

Chapter 6 Mutagenesis of shPRMT5

6.1 Quickchange Reaction to delete 1-50 Amino Acids at the N-terminal End of shPRMT5

The quickchange mutagenesis was carried out on shPRMT5 to cleave 1-51 amino acids at the N terminal. The forward primer sequence was GCCGCGCGGCAGCCATAAACGTGAATTTATTC, and reverse primer sequence was GAATAAATTCACGTTTATGGCTGCCGCGCGGC. To design an appropriate primer, the length of the bases was from 25 to 45 bases. Too long of primers could cause a secondary structure formation and too short of primers could not bind to the template tightly. Another critical aspect was the melting temperature (T_m), which should be higher or equal to 78°C. The formula for calculating T_m is $T_m = 81.5 + 0.41(\%GC) - 675/N$ (N is the primer length in bases and %GC is numbers). The primer contained half sequence from the vector and half sequence from the target gene. A good primer should have a minimum of 40% GC and terminate in C or G bases. In the PCR program, the system was preheated at 95°C for 10 minutes to yield the complete single stranded template DNA to make sure the primers bind well. After DNA amplification in XL1 Blue, one colony containing the correct DNA template with the primers was chosen for the quickchange. The protein with 51-637 amino acids of shPRMT5 was produced using the same methods as previously.

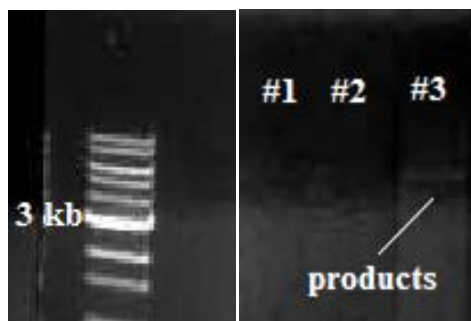


Figure 23. Quickchange with PCR reaction products of 51-637 amino acids of PRMT5. #1, #2, #3 showing three group of solution for quickchange.

In Figure 23, sample #3 DNA templates were transferred into XL1 Blue to be amplified, and three colonies were picked to check the correct sequence. The results showed that colonies of No.1 contained the correct primers of the quickchange. The vector and PRMT5 sequences were consistent.

T7

CATTCCTGCATGACGTCAATCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAG
 ATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGC
GGCAGCCATAAACGTGAATTTATTCAGGAACCGGCGAAAAACCGTCCGGGCCCCGAG
 ACCCGTAGCGATCTGCTGCTGAGCGGCCGTGATTGGAACACCCTGATTGTGGGCAA
 CTGAGCCCGTGGATTCGTCCGGATAGCAAAGTGGAAAAAATTCGTCGTAACAGCGAA
 GCGGCGATGCTGCAGGAACTGAACTTTGGCGCGTATCTGGGCCTGCCGGCGTTTCTG
 CTGCCGCTGAACCAGGAAGATAACACCAACCTGGCGCGTGTGCTGACCAACCATATT
 CATAACGGCCATCATAGCAGCATGTTTTGGATGCGTGTGCCACTGGTGGCACCGGAAG
 ATCTGCGTGATGATATTATTGAAAACGCGCCGACCACCCATACCGAAGAATATAGCGG
 CGAAGAAAAA (The underlined part is the primer.)

The T7 primer was used to check the sequence and the underlined part is the primer which was designed for quickchange. The sequence before the primer was from the vector and the sequence after the primer was the shPRMT5 sequence from 51 amino acid of N-terminal end. Therefore the quickchange of 51-637 was successful.

6.2 Quickchange Reaction to delete 1-200 Amino Acids at the N-terminal End of shPRMT5

shPRMT5 was quickchanged to cleave 1-200 amino acids from N terminal end, which means that the amino acid sequence of the new protein was 201-637 amino acids of shPRMT5. The primer forward was CGCGCGGCAGCCATCGTATTGCGGTGGC, and the primer reverse was GCCACCGCAATACGATGGCTGCCGCGCG.

T7

TAGGCGCCTAGACGTCATTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATAT
 ACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGC
AGCCATCGTATTGCGGTGGCGCTGGAAATTGGCGCGGATCTGCCGAGCAACCATGTG
 ATTGATCGTTGGCTGGGCGAACCGATTAAAGCGGCGATTCTGCCGACCAGCATTTTTC
 TGACCAACAAAAAAGGCTTTCGGTGCTGAGCAAATGCATCAGCGTCTGATTTTTC
 GTCTGCTGAAACTGGAAGTGCAGTTTATTATTACCGGCACCAACCATCATAGCG (The
 underlined part is the primer)

The T7 primer was used to check the sequence and the underlined part is the primer which was designed for quickchange. The sequence before the primer was from the vector and the sequence after the primer was the shPRMT5 sequence from 201 amino acid of N-terminal end. Therefore the quickchange of 201-637 was successful.

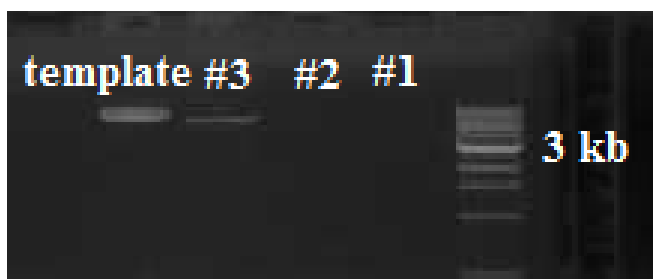


Figure 24. Quickchange with PCR reaction products of 201-637 amino acids of PRMT5. #1, #2, #3 showing three group of solution for quickchange. Template was the original shPRMT5 DNA template.

So in Figure 24, sample #3 showed a single and clear band in the agarose gel, which means high quality of quickchange product of 51-637 protein. 51-637 and 201-637 plasmids were used for expression in the next step.

Chapter 7 Expression and Enzymatic Test of Truncated shPRMT5

7.1 Expression of Truncated shPRMT5 in BL21 (DE3)

After the quickchange reaction of PRMT5, 51-637 and 201-637 fragments of shPRMT5 gene were produced. Figuring out the active site position, which binds to the SAH/SAM, was critical. Because the vector remained unchanged, the 6x-Histag sequence was still at N-terminal end of the new plasmids. The protein expression process was the same as for the previous original PRMT5. BL21 (DE3) bacteria were still used to express the protein and Ni-NTA beads were used to purify his-tagged 51-637 and 201-637 proteins. The molecular weights were changed to around 65 kD of 51-637 protein and 48 kD of 201-637 protein. Through SDS-PAGE bands in Figure 25, 51-637 and 201-637 proteins could be identified. In the picture of SDS-PAGE gel, the bands were at the right positions that proved the protein expression and purification successful. The proteins without 50 amino acids and 200 amino acids at N-terminal end of shPRMT5 were checked whether the active sites existed in them.

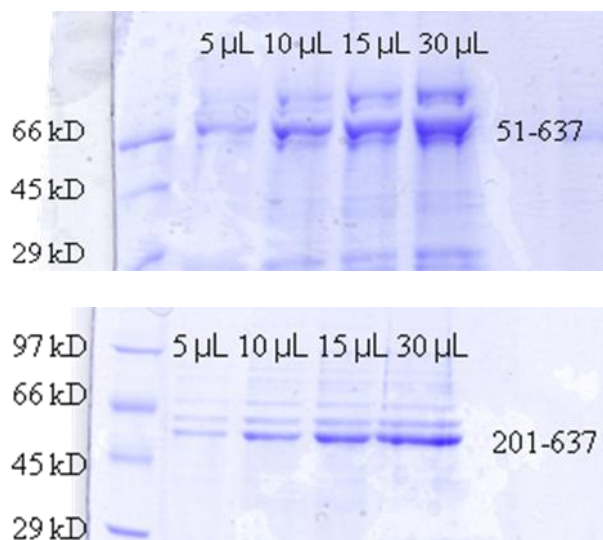


Figure 25. 12% SDS-PAGE gel showing 51-637 and 201-637 protein after purification.

7.2 Radioactive Assay of Truncated shPRMT5

To verify whether 51-637 and 201-637 of shPRMT5 had activity on H4-20, general radioactive assay was set up again with the composition in Table 19. Reaction time of the radioactive assay was 20.5 hours. H4-20 final concentration was 234 μ M and the SAM final concentration was 20 μ M. After the reaction, 50 μ L of each sample and mixture sample was loaded onto P81 papers.

Table 19. Composition of radioactive assay of truncated of shPRMT5 on H4-20.

	H ₂ O	2XRB	Peptide H4-20 (2 mM)	Protein	SAM (400 μ M)	Total
51-637	15 μ L	30 μ L	7 μ L	5 μ L	3 μ L	60 μ L
201-637	15 μ L	30 μ L	7 μ L	5 μ L	3 μ L	60 μ L
No. 2	10 μ L	30 μ L	7 μ L	10 μ L	3 μ L	60 μ L
No. 3	10 μ L	30 μ L	7 μ L	10 μ L	3 μ L	60 μ L
No. 5	10 μ L	30 μ L	7 μ L	10 μ L	3 μ L	60 μ L

Table 20. Results of general test of radioactive assay of truncated PRMT5.

	CPM
51-637	886
201-637	848
No. 2	889
No. 3	793
No. 5	897
mixture	81931

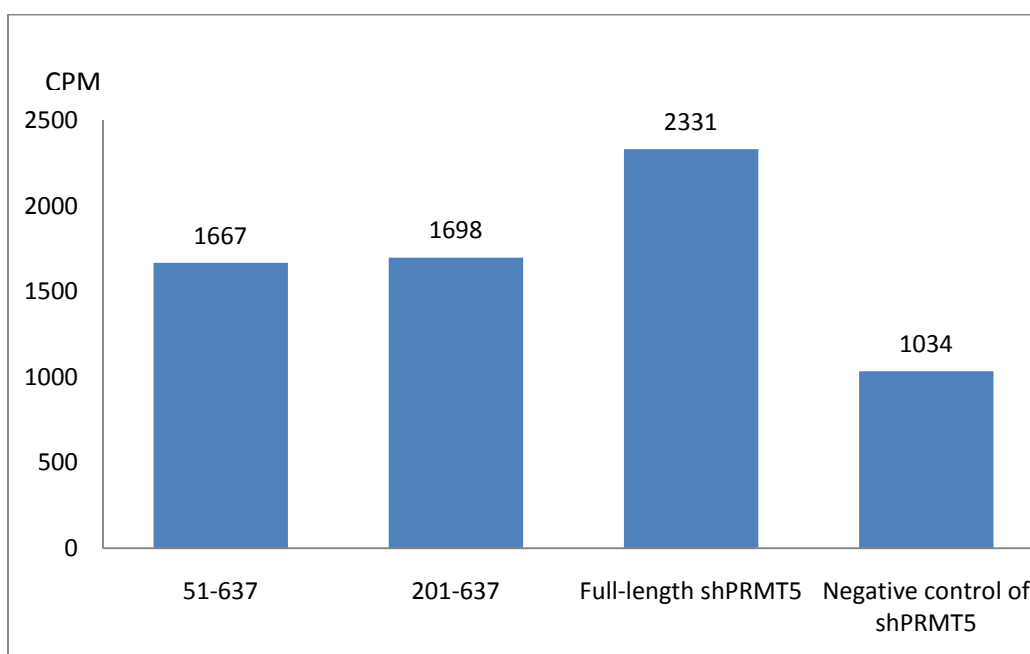
Based on the results of the CPM values shown in Table 10, No.2, No.3 and No.5 had similar CPM values as previous results. No. 3 of had a low concentration but with high activity. Then another general radioactive assay was set up like Table 21 to make sure the results are reliable. Reaction time was still 20.5 hours. H4-20 final concentration was 234 μM and SAM final concentration was 20 μM which were the same as previous assay. 50 μL of each sample and mixture sample was loaded onto P81 papers. The proteins No.2, No.3 and No.5 were not tested this time. One negative control sample was set up as background and full-length shPRMT5 was set up as a control.

Table 21. Composition of radioactive assay of truncated of shPRMT5 on H4-20.

	H ₂ O	2XRB	Peptide H4-20 (2 mM)	Protein	SAM (400 μM)	Total
51-637	15 μL	30 μL	7 μL	5 μL	3 μL	60 μL
201-637	15 μL	30 μL	7 μL	5 μL	3 μL	60 μL
Full-length shPRMT5	10 μL	30 μL	7 μL	10 μL	3 μL	60 μL
Negative control of shPRMT5	20 μL	30 μL	7 μL	—	3 μL	60 μL

Table 22. Results of duplicate general test of radioactive assay of truncated PRMT5.

	CPM
51-637	1667
201-637	1698
Full-length shPRMT5	2331
Negative control of shPRMT5	1034
Mixture	86248

**Figure 26.** The CPM values of 51-637, 01-637, full-length shPRMT5 and negative control of shPRMT5.

In Table 22 and Figure 26, protein 51-637 and 201-637 activities were higher than the negative control of shPRMT5 but lower than full-length shPRMT5. Consequently, full-length shPRMT5 still contains the highest activity on H4-20. However, when first 50 and 200 amino acids were deleted, the activity of 51-637 and 201-637 of shPRMT5 were not decreased dramatically and 201-637 of shPRMT5 had a little higher activity than 51-637 of shPRMT5. So

the results showed that the active site of shPRMT5 was in the region of 201-637 of shPRMT5 and the truncated shPRMT5 had different levels of activity from the full-length shPRMT5. The deletion of a part of amino acids in the N-terminal end of shPRMT5 may change the conformation of the protein, which will influence the activity of the protein on H4-20.

Chapter 8 Bioinformatic Study of PRMT5

8.1 Blast Search of PRMT5

Bioinformatics is a highly effective method to study a specific gene at the sequence and structural levels. Using the databases and the structure prediction servers on the internet are easy and convenient way for understanding the protein structure and finding a relationship between the actual experimental data and protein structure on PDB (protein data bank). BLAST was used to search for similar sequence from the PDB database. The result is in Figure 27.

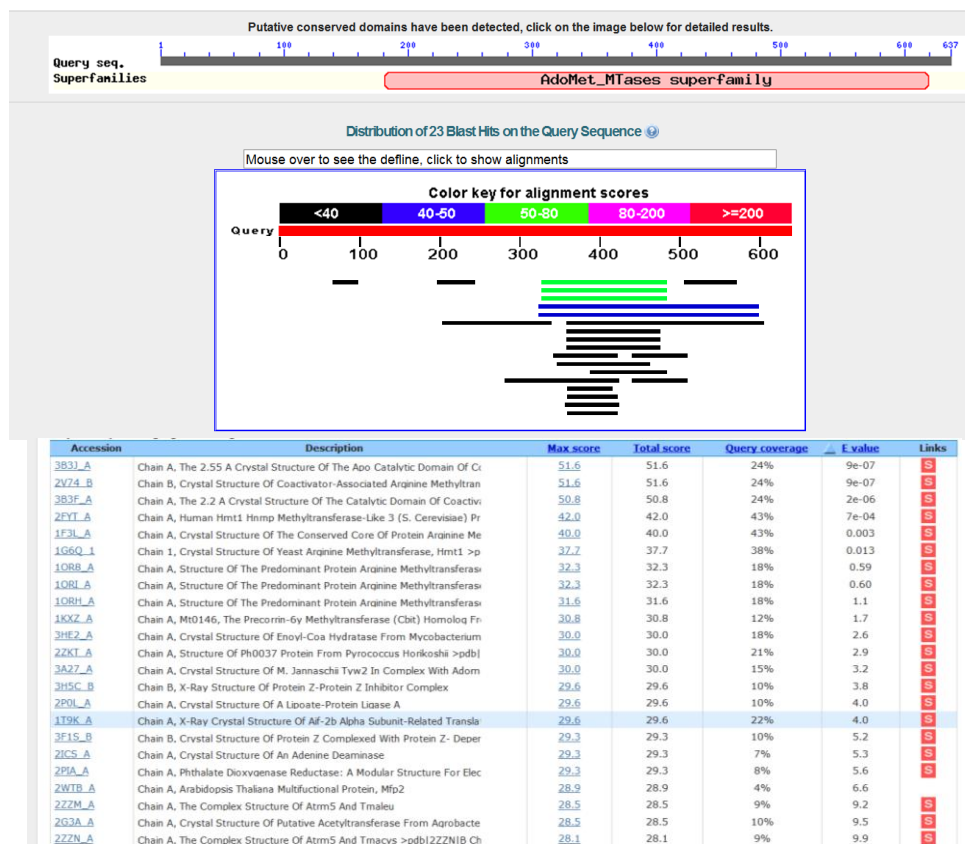


Figure 27. BLAST analysis of PRMT5. (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>)

8.2 CLUSTAL W2 analysis of PRMT5, PRMT1, PRMT3 and CARM1

The result showed that there were few similar sequences with PRMT5. From this result, finding the most similar sequence was the proper choice. Meanwhile, PRMT5 sequence was sent to Dr. Harrison's server of Georgia State University (<http://bmcc3.cs.gsu.edu>). The results from Dr. Harrison's server gave an alignment which showed that PRMT1, CARM1 (PRMT4), PRMT3 were similar. Then PRMT5, PRMT1 (pdb 1ORH), PRMT3 (pdb 1F3L) and CARM1 (pdb 3B3J) sequences were taken CLUSTAL W2 analysis (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

```

1ORH_A|PDBID|CHAIN|SEQUENCE      --RHLFKDKVLDVVGSGTG---ILCMFAAKAGAR--KVIIGIECSSISDYA 108
1F3L_A|PDBID|CHAIN|SEQUENCE      --PHIFKDKVLDVVGCGTG---ILSMFAAKAGAK--KVIADVQSEILYQA  83
3B3J_A|PDBID|CHAIN|SEQUENCE      --HTDFKDKIVLDVVGCGSG---ILSFFAAQAGAR--KIYAVEASTMAQHA 196
PMRT5_A|PDBID|CHAIN|SEQUENCE      EEEKDTNVQVLMVLGAGRGPLVNASLRAAKQADRRRIKLYAVEKNPNAVVI 400
                                     :  :::: :*. * *      .:  ** : . :  *:  :.. .      :

```

Figure 28. CLUSTAL W2 analysis of PRMT5, PRMT1, PRMT3 and CARM1.

8.3 Pattern VLMVLGAGRGPLV in Prosite Server to Figure out the SAH/SAM Binding Site.

From the server result in Figure 28, 360-372 amino acids of PRMT5, which were VLMVLGAGRGPLV, was more similar than the other three sequences. Since the other three proteins were in the same type of PRMTs and the sequences were similar, PRMT1 (pdb 1ORH) was used to compare to PRMT5. Moreover, VLMVLGAGRGPLV sequence was reported as SAM/SAH binding sequence (17). To verify SAM/SAH binding sequence position in PRMT1, VVLDVVGSGTG, which was similar to VLMVLGAGRG of PRMT5, was taken as a pattern to check in the Prosite Server (<http://expasy.org/tools/scanprosite>).

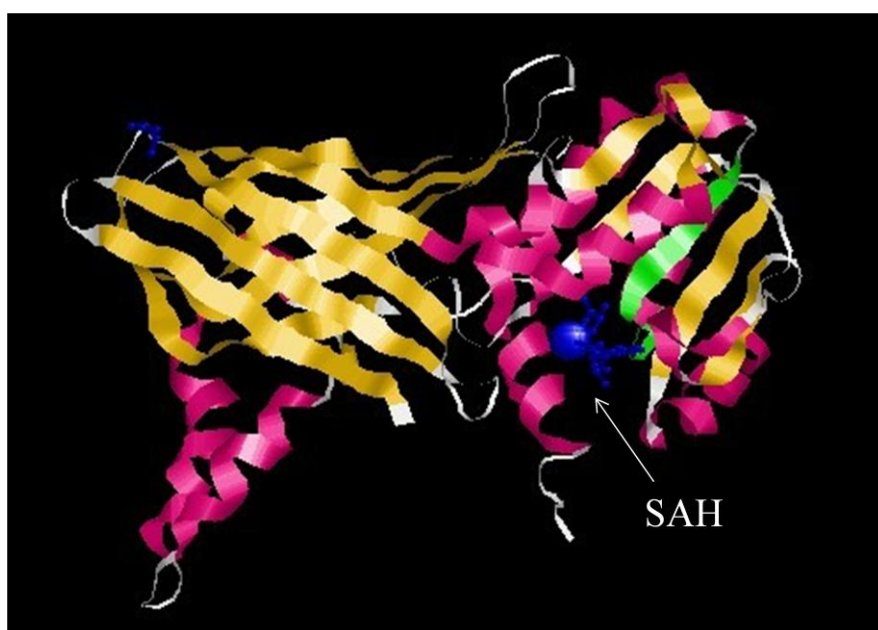
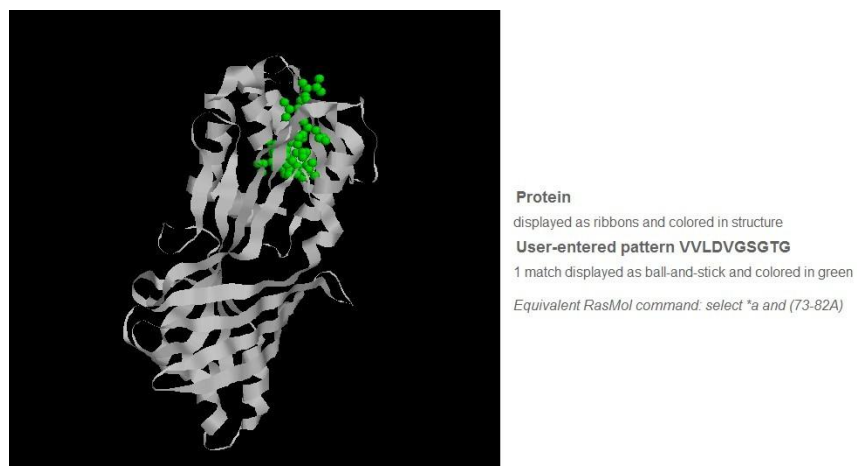


Figure 29. SAH binding site in 3D PRMT1 structure.

8.4 Predict the Structure of PRMT5 SAH/SAM Binding Site

SAM and SAH binding sites are the same. SAH has the same structure as SAM without a methyl group. In PRMT1 (pdb 1ORH), the green color beta-sheet in Figure 29 showed the sequence VVLDVGSGTG position near to the SAH blue ligand. So the sequence VVLDVGSGTG in PRMT1 probably is SAH binding sequence. Then PRMT1 (pdb 1ORH) was

used as the template for PRMT5 structure prediction from Dr. Harrison Server of Georgia State University.

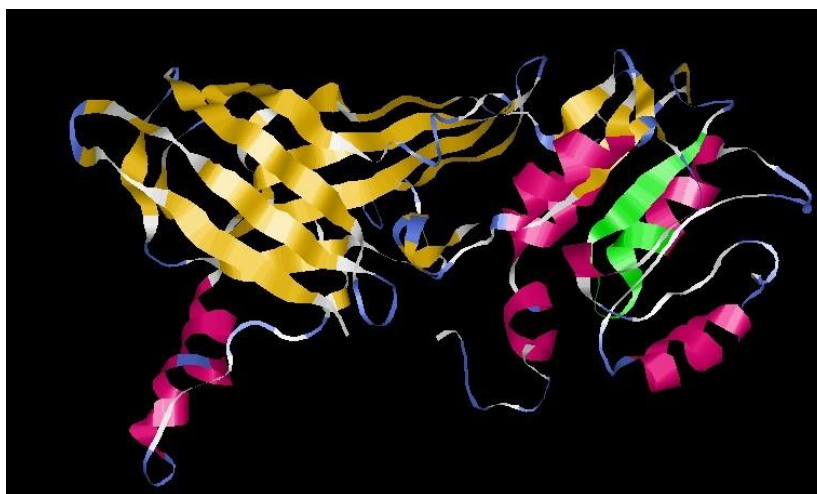


Figure 30. PRMT5 SAH binding site position in the prediction structure which use PRMT1 as a template.

The RasMol showed the predicted structure of PRMT5 with template PRMT1 (pdb 1ROH) in Figure 30. Green part was the sequence VLMVLGAGRGPLV consisting of a beta-sheet and part of an alpha helix. The position was analogous to the PRMT1 SAH binding site in the protein structure. So it is rational to indicate that sequence VLMVLGAGRGPLV in PRMT5 is binding site of SAH/SAM.

8.5 An Analysis SAH Binding Site of Predicted Structure of PRMT5 Using AutoDock

1OR8 is another structure in PDB which is the same as 1ORH of PRMT1. This would provide more straightforward evidence if SAH/SAM was docked in the prediction structure of PRMT5, which use 1OR8 as a template. This analysis is done by Yutao Yang who is a graduate research assistant.

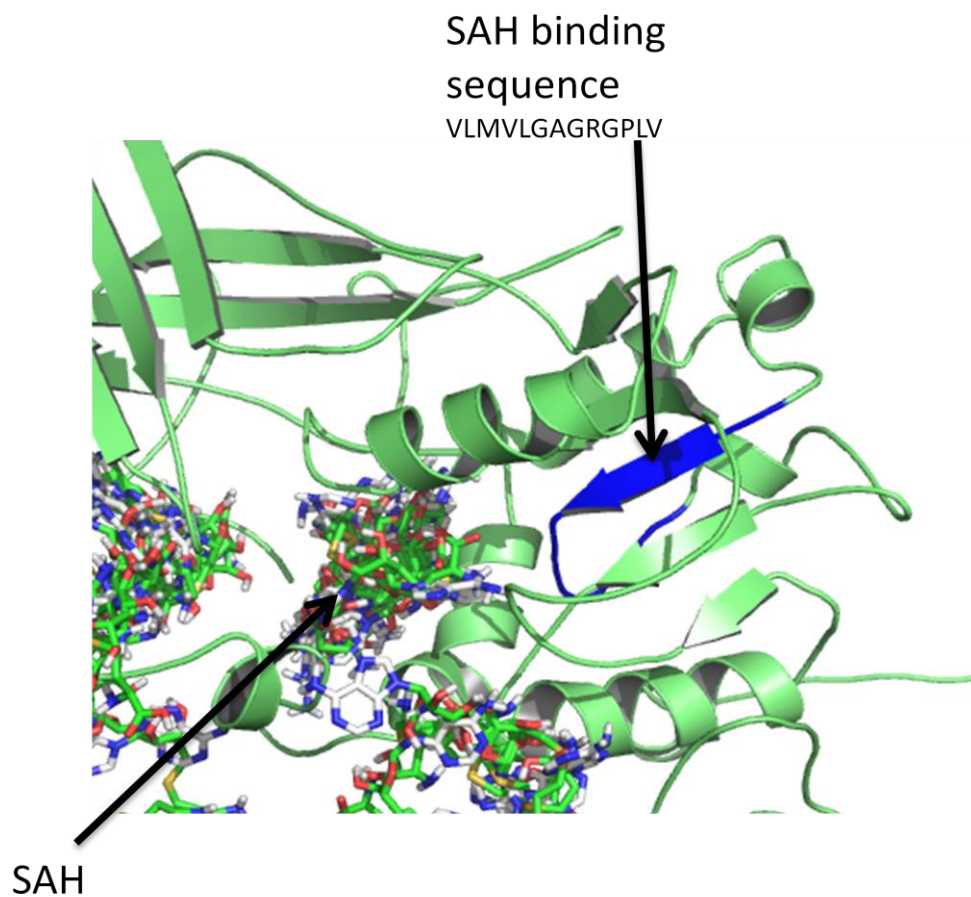


Figure 31. The arrow pointing to the best docking site of SAH in the prediction structure of PRMT5.

In the docking results with AutoDock Version 4.2 software in Figure 31, there were five positions which possibly interact with PRMT5. The high density position indicates the more probable position of SAH. Moreover, the best position was very near to the VLMVLGAGRGPLV region. So 360-372 amino acids in PRMT5 may be the SAH/SAM binding site. However, other SAH/SAM binding sites may also exist. The result was consistent with previous experiment which showed 51-637 and 201-637 amino acids of PRMT5 containing the active site of SAH/SAM.

Chapter 9 Conclusions and Discussion

PRMT5 is a representative and important type II PRMT enzyme. To figure out the enzymatic properties of PRMT5, its function needs to be interpreted in actual cells. Since there was no activity of PRMT5 on H4 and H3 *in vitro* in previous reports, setting up a system of PRMT5 catalysis on H4 and H3 is a priority in studying PRMT5. The original PRMT5 is not expressed well in BL21 (DE3) bacteria. Therefore, a synthetic human PRMT5 (shPRMT5) with optimized DNA sequence the same amino acid that encodes the original PRMT5 was used for expression. In addition, the pET28a vector was used to subclone the shPRMT5 gene. His-tagged shPRMT5 was purified with high efficiency and good quality and it showed a certain activity on H4-20 *in vitro*. However, shPRMT5, which was degraded into multiple bands as shown 12% SDS-PAGE gel, still had certain activity on H4-20. So FPLC was used to separate distinct bands of the degraded proteins. Furthermore, different fractions of shPRMT5 had different activities on H4-20. Since VLMVLGAGRGPLV sequence at 360-372 from the N-terminal end of shPRMT5 was reported as a SAH/SAM binding site in PRMT5, to prove whether this sequence was the SAM/SAH binding site and whether other SAM/SAH binding sites exist is a significant purpose in the experiment. According to the molecular weights and activity, mutagenesis of shPRMT5 was designed to cleave shPRMT5 to different lengths. Then these fractions of shPRMT5 were tested for activity on H4-20. In the present research, amino acids 51-637 and 201-637 from the N terminal end of shPRMT5 were produced and tested for certain activity on H4-20. So, the binding site should be in the 201-637 region of shPRMT5.

In future studies, other fractions of shPRMT5 will be produced and tested for the activity on H4-20. According to the radioactive assay results, the activity seems like not stable enough. Meanwhile, many papers mentioned that PRMT5 is usually recruited to the promoter region of

genes by binding to other proteins or factors to form a complex. It is reasonable to speculate the active site is inside of the protein and other binding factors modify the structure of PRMT5 and enhance or decrease the ability of the active site to interact with histone substrates. This would explain why PRMT5 activity on H4-20 is not stable in vitro.

To sum it up, PRMT5 needs to be studied further at the biochemical level and such a research will be helpful to understand the mechanism of the PRMT enzymes.

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