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Discovery of Novel Cross-Talk between Protein Arginine Methyltransferase Isoforms and Design of Dimerization Inhibitors

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Discovery of Novel Cross-Talk between Protein Arginine Methyltransferase Isoforms and Design

of Dimerization Inhibitors

by

BRANDON CANUP

Under the Direction of Dr. Yujun George Zheng

ABSTRACT

Protein arginine methyltransferase, PRMT, is a family of epigenetic enzymes that methylate arginine residues on histone and nonhistone substrates which result in a monomethylation, symmetric dimethylation or asymmetric dimethylation via the transfer of a methyl group from S-adenosyl-L-methionine (SAM). We discovered a novel interaction between two PRMT isoforms: PRMT1 interacts and methylates PRMT6. In this study site-directed mutagenesis was performed on selected arginines identified from tandem mass spectrometric analysis to investigate major methylation sites of PRMT6 by PRMT1. In combination with radiometric methyltransferase assays, we determined two major methylation sites. Methylations at these sites have significant effects on the nascent enzymatic activity of PRMT6 in H4 methylation. PRMTs have the ability to homodimerize which have been linked to methyltransferase activity. We designed dimerization inhibitors (DMIs) to further investigate the need for dimerization for enzyme activity. Preliminary results suggest that the monomeric form of PRMT1 retains methyltransferase activity comparable to that of the uninhibited PRMT1.

INDEX WORDS: Protein arginine methyltransferase (PRMT), peptide synthesis, histone, dimerization, methylation

DISCOVERY OF NOVEL CROSS-TALK BETWEEN PROTEIN ARGININE METHYLTRANSFERASE ISOFORMS AND DESIGN OF DIMERIZATION INHIBITORS

by

BRANDON CANUP

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Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2013

DEDICATION

I would like to dedicate this to my family for their support and patience. Also to my fiancée Marie Ñunez who supported my educational goals.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Protein arginine methyltransferase (PRMT)

S-Adenosyl-L-methionine (SAM, AdoMet)

S-Adenosyl-L-homocysteine (SAH)

1, 4-Dithiothreitol (DTT)

4-(2-hydroxyethyl)-1-peperazineethanesulfonic acid (HEPES)

Bovine serum albumin (BSA)

Ethylenediaminetetraacetic acid (EDTA)

Isopropyl-β-D-thiogalactopyranoside (IPTG)

Phenylmethanesulphonyl fluoride (PMSF)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Ultraviolet-visble (UV)

Fast protein liquid chromatography (FPLC)

Running Buffer (RB)

2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)

Dimethyl sulfoxide (DMSO)

Dimethylformamide (DMF)

Fluorenylmethyloxycarbonyl (Fmoc)

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS)

Solid phase peptide synthesis (SPPS)

Dimerization inhibitor (DMI)

LIST OF BUFFERS

Cell Lysis buffer: 25 mM Na-HEPES, pH 7.0; 150 mM NaCl; 1 mM MgSO4; 5 % Ethylene glycol; 5 % Glycerol; 1 mM PMSF

Column buffer: 25 mM HEPES, pH 8.0; 500 mM NaCl; 1 mM PMSF; 30 mM Imidazole; 10 % Glycerol

Wash buffer: 25 mM HEPES, pH 8.0; 300 mM NaCl; 1 mM PMSF; 70 mM Imidazole; 10 % Glycerol

Elution buffer: 25 mM HEPES, pH 7.0; 300 mM NaCl; 1 mM PMSF; 10 % Glycerol; 200 mM Imidazole

Dialysis buffer: 25 mM HEPES, pH 7.0; 250 mM NaCl; 1 mM EDTA; 10 % Glycerol; 10 mM DTT

12 % SDS-PAGE Stacking gel (10 mL total volume): 30 % acrylamide; 1.7 mL dd H_2O 6.8 mL; 1.5 M Tris-HCL; pH 6.8 1.25 mL; 10 % SDS 0.1 mL; 10 % APS (ammonium persulfate) 0.1 mL; TEMED 0.01 mL

12 % SDS-PAGE Resolving gel (20 mL total volume): 30 % acrylamide 8.0 mL; ddH_2O 1.7 mL; 1.5 M Tris-HCl pH 8.8 1.25 mL; 10 % SDS 0.2 mL10 % APS (ammonium persulfate); 0.2 mL TEMED 0.008 mL

NZY Media 1 mL LB media: 12.5 µL 1M $MgSO_4$; 12.5 µL 1M $MgCl_2$; 20 µL 20 % Glucose

Reaction Buffer 50 mM HEPES pH 8.0; 50 mM NaCl; 1 mM EDTA; 0.5 mM DTT

Tris-glycine electrophoresis running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3

FPLC running buffer: 25 mM HEPES, pH 7.0; 200 mM NaCl; 1 mM EDTA; 5% glycerol; 1mM DTT

Western blot transfer buffer: 25 mM Tris, 192 mM glycine, 10% methanol

10x Native running buffer: (1 L) 25 mM Tris base, 192 mM glycine

Native sample loading buffer: 100 mM Tris base,10% glycerol, 0.0025% bromophenol blue

1   INTRODUCTION

1.1   Epigenetics

In recent years, it has become apparent that knowing just the genome of an organism does not always dictate what the organism will exhibit phenotypically. Gene expression is regulated by reversible epigenetic mechanisms such as DNA methylation and histone modification. The epigenetic changes can be induced by environment, diet, age, drugs, and heredity. Therefore, the exposure or lack of to these conditions can govern the placement of the epigenetic marks and the packing state of chromatin. As seen in the analysis of identical twins, the environment plays a crucial role that can distinctly alter phenotypes of life [1]. Epigenetic marks have also been shown to be passed down from generations to generations. Animal studies have demonstrated that parents consuming high fat diets can transfer epigenetic marks to their offspring by way of hypomethylation on the Ill3ra2 gene [2]. In addition, epigenetic marks on LG-ABN can alter serotonin release and NGFI-A expression causing shifts in maternal behavior in mice towards its pups [3]. In all the instances epigenetic marks are altered or added to induce physiological and neurological differences, as well as, regulating the expression of genes to give rise to its cellular identity. This idea has given rise to the "one genome many identities" concept during early stages of embryonic development. Therefore, deregulation of epigenetic patterns can lead to serious disease states. More and more evidence show epigenetic enzymes are important novel molecular targets in disease prevention and therapy.

1.2   Histone modifications

Chromatin is found in eukaryotic cells as a DNA and protein complex (Figure 1.1). There is ~147-bpDNA is wrapped around a histone octamer consisting of two sets of H4, H3, H2A, and H2B to form the nucleosome. The nucleosome structures are connected by a continuous strand of DNA linked

by H1 that resembles beads-on-string. This open conformation allows for easy access to gene sequences

for expression. The nucleosomes are tightly wound to form 30 nm coils called solenoids that do not al-

low for the access to the underlying gene sequences thereby repressing gene expression. The open state

of chromatin is referred to as euchromatin or active state. The condensed form of chromatin is called

heterochromatin or inactive state. The solenoid fibers are then tightly folded to form the chromosome.

Unfolding and refolding of these structures are dictated by various epigenetic modifications on the DNA

and histones.
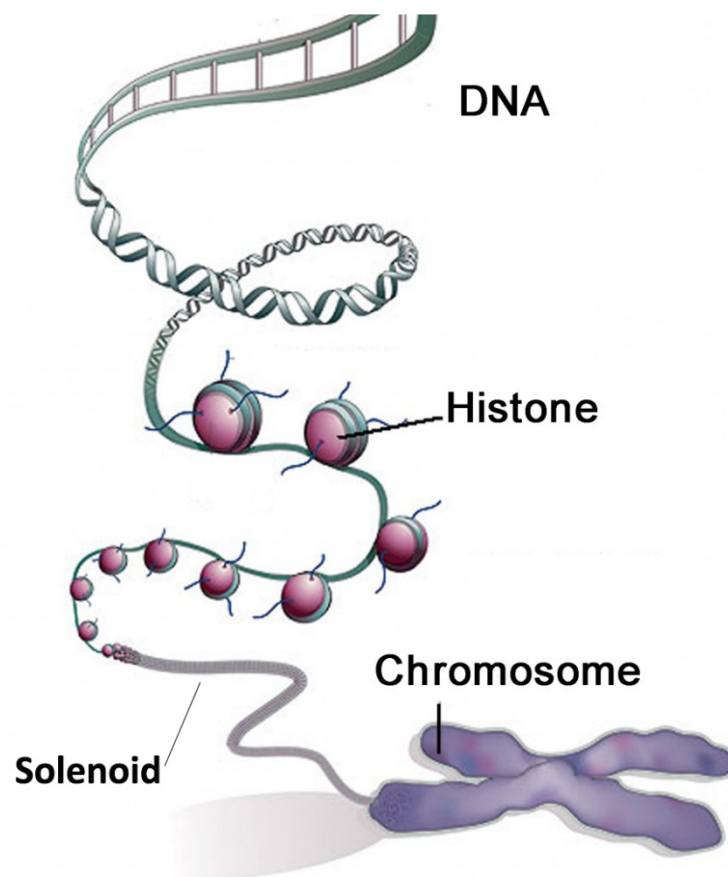
Figure 1.1- Packing of DNA into the chromosome[4]

Histones undergo multiple posttranslational modifications (PTMs). The modifications usually oc-

cur on the amino terminal tails; however, more reports have emerged that even the fold domain his-

tones can be modified. These modifications include lysine acetylating, methylation, and ubiquitination,

arginine methylation, serine and threonine phosphorylation. Modifications occur in unique patterns in

order to induce different functions. For example, phosphorylation of S31 on H3.3 is believed to be important in stages of mitosis, but does not occur in later phases when S28 is phosphorylated during chromosome condensation [5]. Lysine residues on H3 can be modified by multiple PTMs. Transcriptional activation occurs with H3 K9 and K4 di-/tri-methylation and repression occurs with H3K27 trimethylation[6]. Interesting, GCN5 acetylation at H3 K9 and K14 are important for forming complexes with transcription factors[7]. There exists extensive cross talk between PTM modifications. Acetylation of H3 K18 and K23 allows for CARM1 H3 R17 methylation in vivo [8]. In another case, phosphorylation at S217 of CARM1 abolishes methyltransferase activity during mitosis [9]. The mechanisms of PTM regulation of modification levels are not completely understood. Further study of the complex interactions of epigenetic marks will lead to greater understanding of how defects emerge.

1.3 Protein arginine methyltransferase

Protein arginine methyltransferases (PRMTs) are a class of epigenetic enzymes that facilitate the transfer of methyl group from S-adenosyl-L-methionine (SAM) to the guanidino group of arginine residues of histone and a variety of non- histone substrates. Currently, only 9 PRMTs have been discovered in mammalian cells. The PRMT family can separated into 3 types based on the final methylated form of the arginine residue as shown in Figure 1.2. Type I PRMTs consist of PRMT-1, -2, -3, -4, -6, and -8 which can produce monomethyl and symmetric dimethyl arginines.[10-14] Type 2 PRMTs consist of PRMT- 5, -7, and -9 which produce monomethyl and asymmetric dimethyl arginines.[14-16] Lately it has been reported that PRMT-7 produces only the monomethyl arginine.[17]
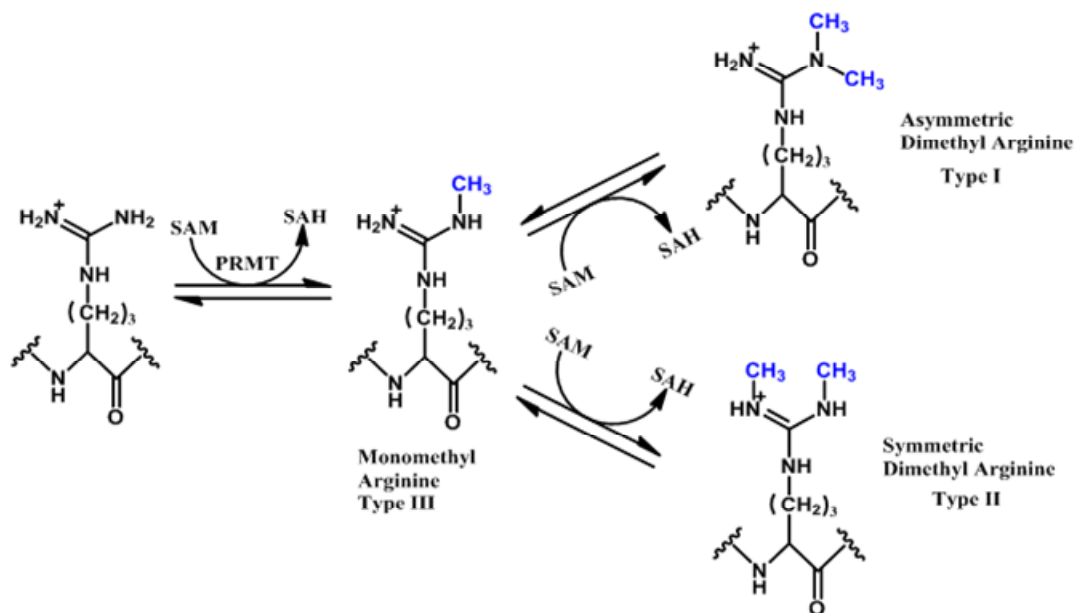
Figure 1.2- Protein arginine methylation mediated by PRMTs

PRMT1 and PRMT6 are both type I PRMTs. PRMT1 is the predominant type I PRMT in vivo and knockdown of PRMT1 has shown to be lethal.[18, 19] Knockdown of PRMT6 results in proliferation defects in cells but not lethality.[20] The reported mechanism for PRMT1 is a rapid equilibrium sequential mechanism.[21] The kinetic mechanism for PRMT6 is still not fully confirmed and is between ordered sequential and random sequential.[22, 23] PRMT1 and PRMT6 play similar roles in cell movement, repair, transcriptional regulation, human immunodeficiency virus pathogenesis, and are found to be dysregulated in several cancer lines.[10, 24-28] With the diverse overlap of these two enzymes it is interesting that they can interact. In this study we analyzed PRMT1-PRMT6 interaction and discovered a novel crosstalk of PRMT isoforms.

PRMTs share structural similarity. The dimerization arm is conserved throughout the PRMT family suggesting that dimerization is important to function[29]. The AdoMet binding domain and unique β barrel are also conserved for essential SAM binding [29-31]. Previously mutations of conserved residues that reside on the dimerization interface reduce methyltransferase activity and complete deletion

of the dimerization arm leads to complete loss in activity[29]. In our study we show that this may not be the case. Preliminary data for the isolation of a monomeric species of PRMT1 using dimerization inhibitors show similar methyltransferase activity to that of the uninhibited PRMT1. This provides novel insights that perhaps the dimerization of PRMTs is critical for stability but not necessarily for activity. Design if inhibitors that target this dimerization could provide chemical probes that interrogate the role of dimerization in PRMT catalysis.
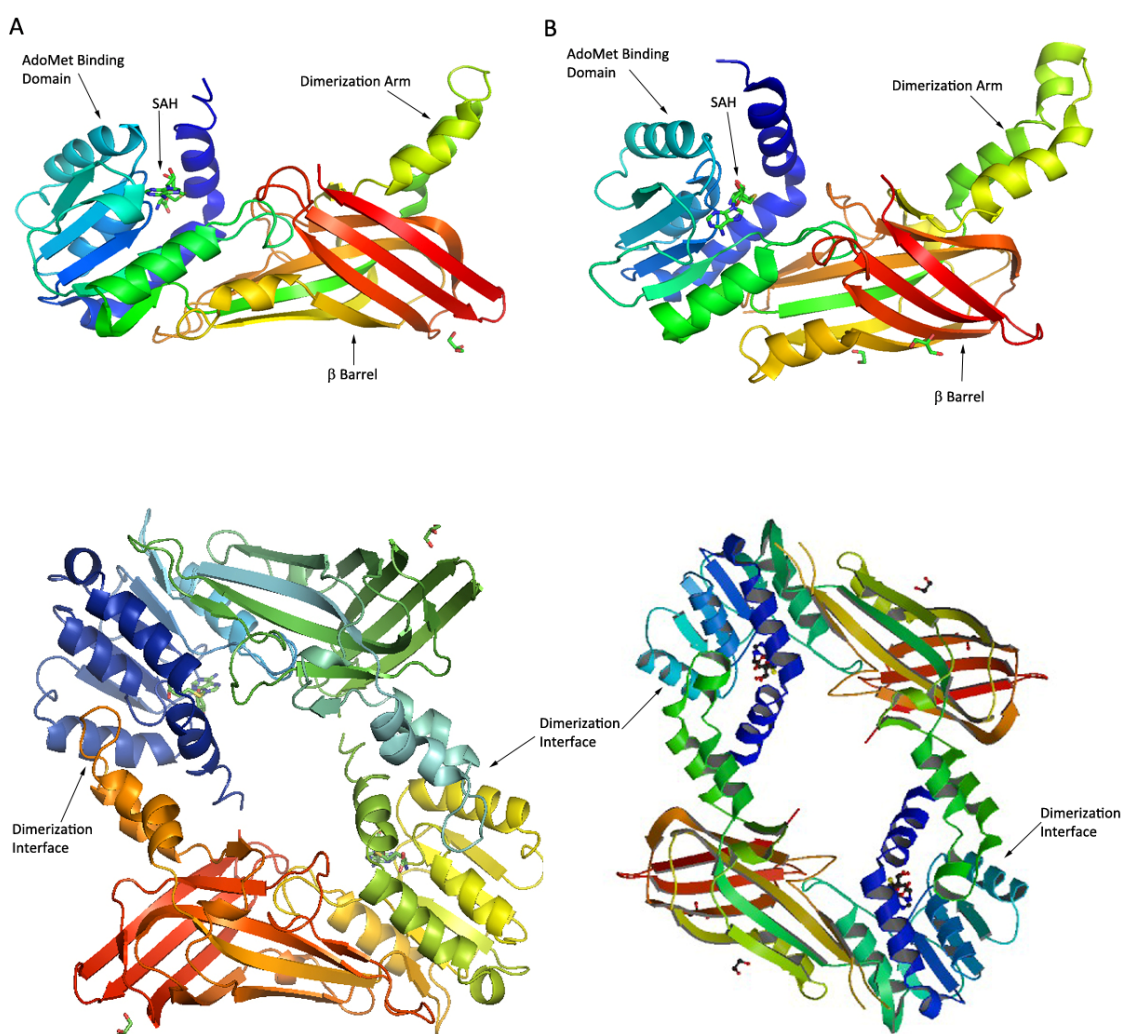
Figure 1.3- Crystal structures of PRMT1 and PRMT6 monomer and dimer. (A) PRMT1 crystal structure (pdb:1ORH) shown as both a monomer and dimer. The dimerization interface consists of a hydrophobic pocket utilizing the dimerization arm and AdoMet binding domain. (B) Un-

published PRMT6 crystal structure (pdb:4HC4) missing 40 residues from the full sequence on the N-terminal region. Like PRMT1 it shows similar structures including the beta barrel, AdoMet binding domain and dimerization arm.

1.4   Previous work and goals

Previously our laboratory discovered a novel interaction between PRMT1 and PRMT6. Prior experiments were primarily performed by Dr. You Feng. Coimmunoprecipitation was performed to demonstrate the PRMT isoform interaction occurring In vivo (Figure 1.4). HEK-293T cells were used to cotransfect HA-PRMT1 and Myc-PRMT6. Anti-HA agarose beads were used to immunoprecipitate the cell lysate and immunoblotting with anti-PRMT6 or anti-HA antibodies were used for the eluents. Cell lysates of cotransfection were also immunoprecipitated with anti-HA antibody or mouse IgG followed by Protein A/G Plus agarose beads. The eluents were also immunoblotted with anti-PRMT6 or anti-HA antibodies. Myc-PRMt6 was transfected into HEK-293T cells. The cell lysates were immunoprecipitated with anti-Myc antibody or mouse igG. Anti-PRMT1 or anti-Myc antibodies were used to immunoblot the immunoprecipitates. PRMT6 was detected in the HA-PRMT1 pull down. PRMT1 could be detected in the Myc-PRMT6 pull down fraction. This showed that PRMT1 and PRMT6 do interact in the cell

(A) Cotransfection, IP with HA-conjugated beads

(B) Cotransfection, IP with protein A/G plus agarose beads

(C) Single transfection, IP with protein A/G plus agarose beads
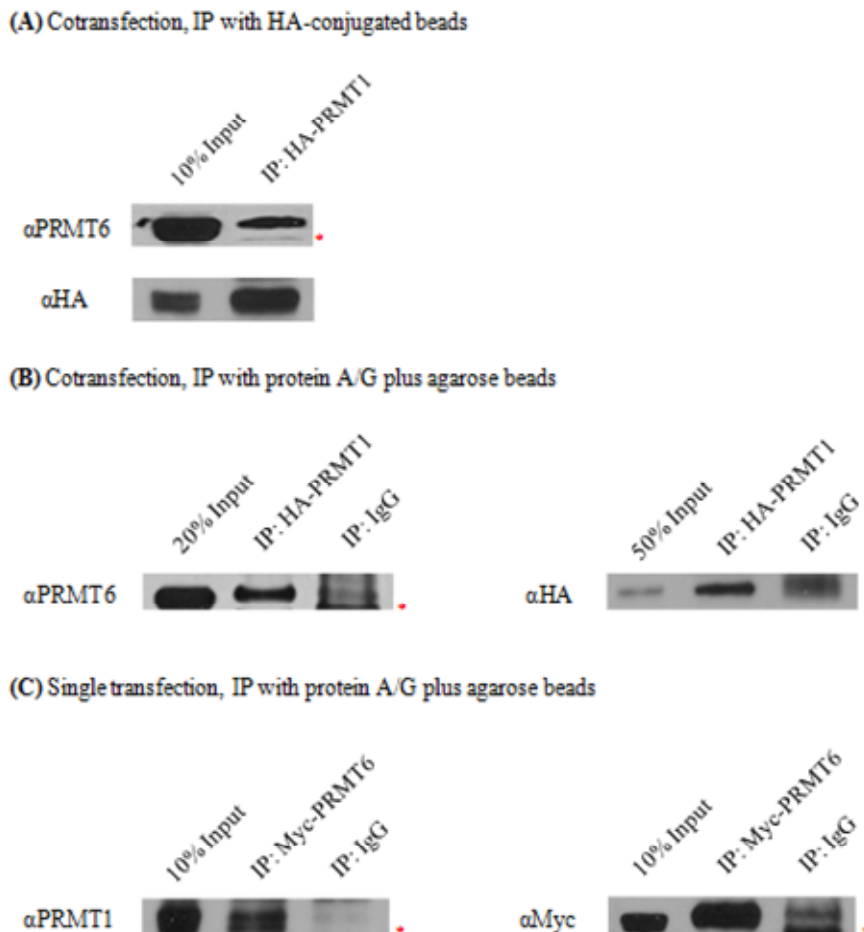
Figure 1.4- Immunoprecipitation of HA-PRMT1 and Myc-PRMT6. (A) HEK-293T cells were used to cotransfect HA- PRMT1 and Myc-PRMT6 and anti-HA agarose beads were used for immunoprecipitation of cell lysate. Anti-PRMT6 or anti-HA antibody were used to immunoblot the eluents. (B)) HEK-293T cells were used to cotransfect HA- PRMT1 and Myc-PRMT6 and anti-HA antibody or with mouse IgG were used for immuno precipitation with Protein A/G Plus agarose beads. Anti-PRMT6 or anti-HA antibody were used to immunoblot the eluents. (C) Myc-PRMT6 was transfected into HEK-293T cells.Anti-Myc antibody or with mouse IgG were used to immunoprecipitate the cell lysate. Anti-PRMT1 or anti-Myc antibody was used to immunoblot the eluents. The nonspecific bands are labeled with asterisks.

Another experiment performed was to investigate if the interaction between PRMT1 and

PRMT6 is similar to the reported PRMT1 and PRMT2 interaction by froming stable heterodimers (Figure

1.5). PRMT1 was incubated with varied concentrations of PRMT6 in the presence of 0.1% Triton X-100

overnight. The samples were crosslinked with 0.025 % glutaraldehyde for 5 minutes and were resolved

on an 8% SDS-PAGE. Western blot detection used primary antibody (anti-PRMT1 rabbit polyclonal IgG)

and secondary antibody (goat anti-rabbit IgG-HRP) for PRMT1 detection. PRMT6 was incubated

with/without PRMT1 in the presence of 0.1% Triton X-100 overnight. A sample of PRMT1 was also incu-

bated by itself. The experimental procedure for PRMT6 is the same to that of PRMT1. The antibodies

used for Western blot detection were anti-PRMT6 mouse IgG and goat anti-mouse IgG-HRP. The West-

ern blot did not show the formation of heterodimers. In the presence of increasing PRMT1 or PRMT6 did

not alter the oligomeric states of each PRMT.
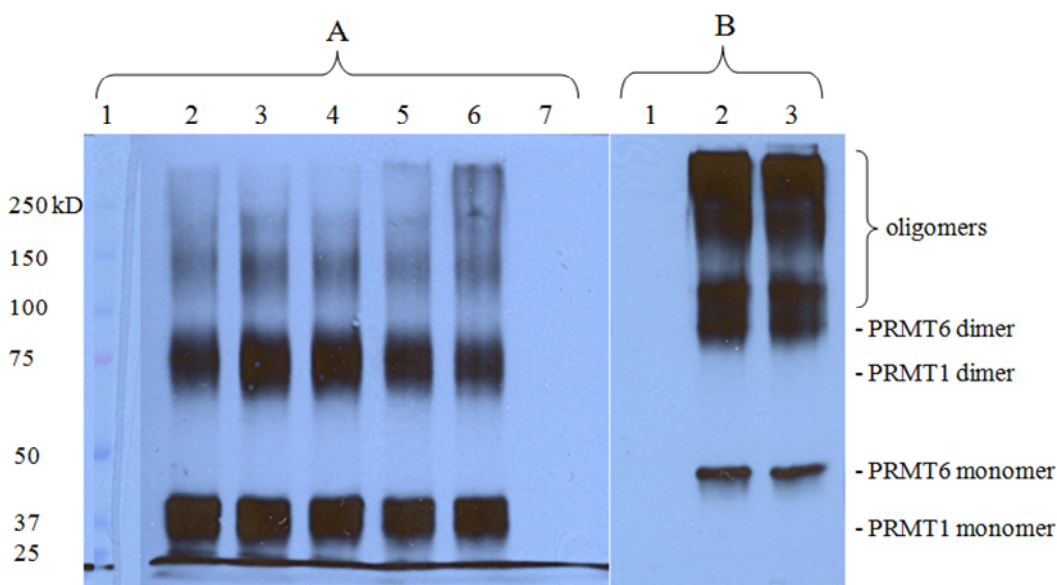
Figure 1.5- Western Blot of PRMT1 and PRMT6. (A) 1) marker; 2) PRMT1; 3) PRMT1 + 0.012 μM PRMT6; 4) PRMT1 + 0.024 μM PRMT6; 5) PRMT1 + 0.10 μM PRMT6; 6) PRMT1 + 0.1 μM PRMT5; 7) 0.10 μM PRMT6 as negative control. (B) . 1) 0.024 μM PRMT1 as negative control; 2) 0.024 μM PRMT1 + 0.024 μM PRMT6; 3) 0.024 μM PRMT6.

Based on a radiometric assay, the interaction between PRMT1 and PRMT6 was investi-

gated further and we found that PRMT1 can methylate PRMT6 (Figure 3.13). To elucidate the

methylation sites on PRMT6 by PRMT1 tandem-MS/MS analysis was conducted by Dr. You Feng.

PRMT6 was incubated with SAM and PRMT1 for 3 hours at 30°C. The reactions were quenched

with SDS loading dye and resolved on a 10% SDS-PAGE. The PRMT6 band was cut out, washed in

destain buffer, and vacuum dried. The dried gel was sent to UAB Proteomics & Mass Spectrome-

try Facility (Birmingham, AL 35294) for in gel digestion (Trypsin) and LC-MS/MS detection. Five

Arg residues were found to be methylated based on the result of this analysis. R82 and R228

were dimethylated and R29, R106, and R174 were monomethylated (Appendix: Figure SI-1).

Primers were designed to mutate these residues.

The goals of this project are to investigate in detail how PRMT1 methylates PRMT6. The

mutational study will ultimately provide the major target for PRMT1 mediated methylation of

PRMT6. To understand this interaction will provide information on how these enzymes are regu-

lated in vivo. To discover if PRMT1 mediated methylation could regulate PRMT6 activity and

substrate specificity.

2 EXPERIMENTAL METHODS

2.1 PRMT1 and PRMT6 Experiments

2.1.1 Expression and purification of PRMT1

His-PRMT1 were expressed using 50 μL *E. Coli* BL21(DE3) cells. The solution was inoculated in ice for 20 minutes, heated at 42°C for 30 seconds, and placed in ice for 2 minutes. Preheated LB media was added to the tubes then incubated for 1 hour at 30°C. Incubated samples were spread on preheated agar plates treated with kanamycin and were left overnight to colonize. Four colonies were collected and inoculated in separate 8 ml LB broths treated with 1000x kanamycin overnight. Seeds were collected and stored in -80°C. The 8 ml solutions were added to two 1 L LB broth with 1 ml kanamycin. The large cultures were incubated at 37°C with shaking till O.D. was between 0.6-0.9. The temperature was then decreased to 16°C and allowed to shake overnight. Protein expression was induced with the addition of IPTG at a final concentration of 0.3 mM. After protein expression, the large cultured were centrifuged at 5,000 RPM at 4°C for 10 min. After discarding the supernatant, each of the cell pellets were suspended in 25 mL lysis buffer. The cell lysis of the membranes was performed using a French Press. The lysate was centrifuged at 14,000 RPM for 30 minutes at 4°C. A sample of the supernatant and pellet were collected for SDS-PAGE. A column was used with 5 mL Ni-charged His6x-tag binding resin (Novagen) to purify the protein. The volume of nickel beads used was increased to 7-8 mL because they were recovered nickel beads with less efficiency. The beads were equilibrated with column equilibrium buffer five times. The supernatant was loaded onto the column and allowed to shake for an hour. A sample of the flow through was taken for SDS-PAGE. Equilibrium buffer was run through the column 5 times. Column washing buffer was run through the column slowly. The protein was eluted using elution buffer. Different elution fractions were taken as samples to track protein elution along with samples of the nickel beads before and after elution for SDS-PAGE. The eluted proteins were concentrated to 15 ml

using 10,000-NMWL centrifugal filters (Millipore) tubes at 5,000 rpm at 4°C. The concentrated protein solutions were then placed into dialysis bags and dialyzed overnight against the storage buffer. The protein solutions were concentrated further to 2 ml using recovered centrifuge tubes and placed in dialysis as before in a Slide-a-Lyzer Dialysis Cassette 10,000 MW. A 12% SDS-PAGE gel was run with the samples collected. The protein concentration was determined using Bradford Assay. The final protein samples were aliquot and flash frozen and stored in the -80°C.

$$Concentration\ \left(\frac{mg}{ml}\right) = \frac{Average\ Protein\ A_{595}}{Average\ BSA\ A_{595}} \times 2$$

$$Concentration(\mu M) = Concentration(\frac{mg}{ml}) \times \frac{10^6}{Protein\ MW}$$

Eqaution 2.1- Bradford assay eqautions for concentration

2.1.2   *Site directed mutagenesis of PRMT6*

Primers containing the mutations were designed based on Tandem MSMS data and sent to Integrated DNA Technologies to be prepared. The PRMT6 primers were previously designed You Feng for PRMT6 mutants R29K, R82K, R106K, R228K, R174K, M60L, and M166A. The DNA template used was His6×PRMT6-pET28a(+). Stocks of 40 ng/µl of the primers and 20 ng/µl were prepared for the PCR experiment. The forward and reverse primers were loaded into small tubes in ice containing native plus pfu buffer, dNTP, and various amounts of DNA template (Table 1.1). Prior to adding native pfu polymerase the tubes were placed in the PCR for 5 min at 95°C to ensure initial DNA melting. The native pfu polymerase was added to each tube after initial heating. The tubes were placed into the PCR for a set number of thermo cycles overnight for optimal amplification as shown (Table 1.2). The amplified DNA plasmids were then run on a 1% agarose and scanned using a UV illuminator to check if the desired DNA plasmid was obtained. The acquired plasmids were digested with 1 µl DPn1 remove the original plasmid for 1 hour at 37°C and stored in the -20°C till XL1-Blue transformation.

Table 2.1- Site directed mutagenesis tubes for PCR

	Tube 1	Tube 2	Tube 3
Reagent	μl	μl	μl
10X Native plus pfu buffer	5	5	5
DNA template (20 ng/μl)	1	2	5
Primer Fw (40 ng/μl)	3	3	3
Primer Rev (40 ng/μl)	3	3	3
10 mm dNTP	1	1	1
ddH$_2$O	36	35	32
Native pfu polymerase	1	1	1

The final volume for each tube was 50 μl. The varied loading amount of DNA template is to pro-

vide various conditions for mutagenesis to occur.

Table 2.2- Quick change PCR program for thermocycles

Quickchange PCR Program		
Cycle	T^{o}C	Time
1	95	30 sec
2	95	30sec
3	55	1 min
4	68	13 min
5	2-5 repeat	17 times
6	4	Forever
7		End

The cycles used are standard practice for site directed mutagenesis procedures. The elongation

step was calculated by using 2 min/kb.

2.1.3   Transformation into XL1-Blue, DNA extraction, and DNA sequencing

Plasmids, 1 μl each, were transformed with 50 μL XL1 Blue cells. The initial plasmid and cell solu-

tion was placed on ice for 30 minutes, heat shocked at 42°C for 45 seconds, and placed on ice once more

for 2 minutes. Then 0.5 μl pre-heated NZY-broth was added to each and was set to rotate at 37°C for 1

hour. At the same time, kanamycin agar plates were placed to preheat. For each sample 80 μl and 100 μl

were spread on two of the preheated plated and left overnight at 37$^\text{o}$C. Colonies were collected the next day and were placed into inoculation tubes containing 4 ml LB media with 4 μl kanamycin. The tubes were rotated overnight at 37$^\text{o}$C. The next day the solutions were centrifuged at 3,000 rpm at 4$^\text{o}$C and the pellet was prepared for DNA extraction.

DNA extraction was performed using a Promega DNA purification system kit. The pellets were resuspended using 250 μl resuspension solution and transferred to autoclaved 1.5 ml eppendorf tubes. Lysis solution, 250 μl, was added to each tube and inverted 4 times to mix. Alkaline protease solution, 10 μl, was added and allowed to incubate for 5 min at room temperature. Neutralization solution, 350 μl, was added and centrifuged at top speed for 10 min at room temperature. The supernatant was decant-ed into wizard SV minicolumns (spin columns) and centrifuged for 1 min at room temp. Two sets of wash solutions 750 μl and 250 μl were separately centrifuged for 1 min and 2 min each. The DNA was extract-ed from the spin column filter using nuclease free water by centrifuging for 1 min after inoculating for 2 min. The concentrations were determined using eq 1 with a UV-Vis at 260 nm using 98 μl ddH$_2$O and 2 μl of DNA sample. Samples were sent to *Genscript Inc* for DNA sequencing to determine which colonies contained the correct DNA sequence. The sequences obtained were compared with the original se-quence (figure 1.1) using *BLAST* software on the NCBI website. The correct sequences were then carried onto *E. Coli* BL21(DE3) transformation for protein expression.

$$[DNA] = Abs_{260} \times 0.05 \times 50$$

Eqaution 2.2- DNA concentration equation

```
          10          20          30          40          50          60
MSQPKKRKLE SGGGGEGGEG TEEEDGAERE AALERPRRTK RERDQLYYEC YSDVSVHEEM

          70          80          90         100         110         120
IADRVRTDAY RLGILRNWAA LRGKTVLDVG AGTGILSIFC AQAGARRVYA VEASAIWQQA

         130         140         150         160         170         180
REVVRFNGLE DRVHVLPGPV ETVELPEQVD AIVSEWMGYG LLHESMLSSV LHARTKWLKE

         190         200         210         220         230         240
GGLLLPASAE LFIAPISDQM LEWRLGFWSQ VKQHYGVDMS CLEGFATRCL MGHSEIVVQG

         250         260         270         280         290         300
LSGEDVLARP QRFAQLELSR AGLEQELEAG VGGRFRCSCY GSAPMHGFAI WFQVTFPGGE

         310         320         330         340         350         360
SEKPLVLSTS PFHPATHWKQ ALLYLNEPVQ VEQDTDVSGE ITLLPSRDNP RRLRVLLRYK

         370
VGDQEEKTKD FAMED
```

Figure 2.1- Full sequence of PRMT1. Sequence of hPRMT6 used as a reference for analyzing mutant sequences. Mutations are highlighted in the sequence.

2.1.4 Expression and purification of PRMT1, PRMT6 and PRMT6 mutants

PRMT6 mutants and PRMT6 wild type were expressed using 50 μL *E. Coli* BL21(DE3) cells. The solution was inoculated in ice for 20 minutes, heated at 42°C for 30 seconds, and placed in ice for 2 minutes. Preheated LB media was added to the tubes then incubated for 1 hour at 30°C. Incubated samples were spread on preheated agar plates treated with kanamycin and were left overnight to colonize. Four colonies were collected and inoculated in separate 8 ml LB broths treated with X1000 kanamycin over night. Seeds were collected and stored in -80°C. The 8 ml solutions were added to two 1 L LB broth with 1 ml kanamycin. The large cultures were incubated at 37°C with shaking till O.D. was between 0.6-0.9. The temperature was then decreased to 16°C and allowed to shake overnight. Protein expression was induced with the addition of IPTG at a final concentration of 0.3 mM. After protein expression, the large cultured were centrifuged at 5,000 RPM at 4°C for 10 min. After discarding the supernatant, each

of the cell pellets were suspended in 25 mL lysis buffer. The cell lysis of the membranes was performed

using a French Press. The lysate was centrifuged at 14,000 RPM for 30 minutes at 4°C. A sample of the

supernatant and pellet were collected for SDS-PAGE. A column was used with 5 mL Ni-charged His6x-tag

binding resin (Novagen) to purify the protein. The volume of nickel beads used was increased to 7-8 mL

because they were recovered nickel beads with less efficiency. The beads were equilibrated with column

equilibrium buffer five times. The supernatant was loaded onto the column and allowed to shake for an

hour. A sample of the flow through was taken for SDS-PAGE. Equilibrium buffer was run through the

column 5 times. Column washing buffer was run through the column slowly. The protein was eluted us-

ing elution buffer. Different elution fractions were taken as samples to track protein elution along with

samples of the nickel beads before and after elution for SDS-PAGE. The eluted proteins were concen-

trated to 15 ml using 10,000-NMWL centrifugal filters (Millipore) tubes at 5,000 rpm at 4°C. The concen-

trated protein solutions were then placed into dialysis bags and dialyzed overnight against the storage

buffer. The protein solutions were concentrated further to 2 ml using recovered centrifuge tubes and

placed in dialysis as before in a Slide-a-Lyzer Dialysis Cassette 10,000 MW. A 12% SDS-PAGE gel was run

with the samples collected. The protein concentration was determined using Bradford Assay. The final

protein samples were aliquot and flash frozen and stored in the -80°C.

2.1.5 *PRMT1 and PRMT6 methylation and interaction*

The radioactive assays were performed with 20 μM ^{14}C$^-$SAM and a reaction buffer (HEPES pH 8:

200 mM, NaCl: 200mM, EDTA: 4 mM, and DTT: 2 mM). PRMT6 (1μM) methylation was examined by it-

self, in the presence of 3μM PRMT1 and inactive E153Q-PRMT1, and with 3 μM H3-3 protein as a sub-

strate. The initial reaction mixture containing ^{14}C$^-$SAM, 2RB, and ddH$_2$O was incubated with PRMT1,

E153Q-PRMT1, and H3-3 for 5 minutes at 30°C. PRMT6 was added last and was incubated for 1 hour at

30°C. A control was performed with 3μM PMT1 without PRMT6. The reactions were quenched using 5x

SDS loading dye and resolved on a 12% SDS gel. Staining and distaining of the gel was performed prior to

the 2 hour drying period to see loading amounts. After drying the gel was stored in a Phosphor-image cassette for 48 hours before scanned on a Typhoon scanner.

2.1.6   Methylation rate analysis of PRMT6 with and without PRMT1 or PRMT1-E153Q

5 μM PRMT6 was incubated with 2RB, 30 μM ^{14}C SAM, and with and without 0.4 μM E153Q-PRMT1 or 0.4 μM PRMT1 for a reaction period from 0-120 min. At each time point the reactions were quenched with 7.5 μl 5x SDS loading dye. The samples were resolved on a 16% SDS gel using 200V. Staining and distaining were performed to ensure proper loading amounts and to remove any excess impurities. The gel was dried for 2 hours using filter paper and vacuum and placed in a phosphor-imaging cassette for 48 hours. The image was scanned on a Typhoon scanner.
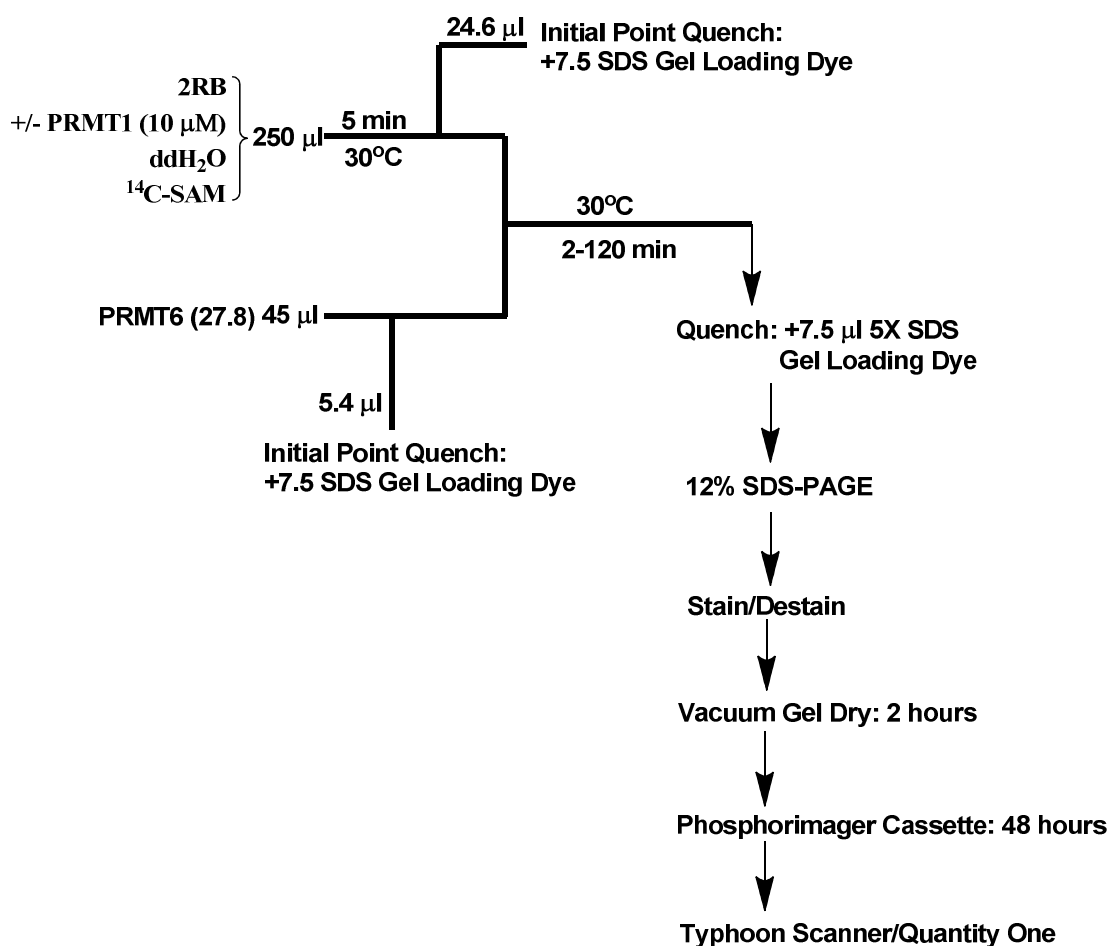
Scheme 2.1- Radioactive Methylatransferase Assay for Rate

2.1.7   PRMT6 wt and mutant methylation via PRMT1 and automethylation

The PRMT6 wt and mutants were diluted to 10 μM and were allowed to incubate at 30°C for 5

mins. A reaction mixture was prepared containing 4 RB (HEPES pH 8: 200 mM, NaCl: 200mM, EDTA: 4

mM, and DTT: 2 mM), ddH$_2$O, ^{14}C -SAM, and +/- PRMT1 to test both methylation strategies. The two so-

lutions were combined after the initial 5 min inoculation and left to react for 0-2 hours at 30°C. The re-

action was quenched using 7.5 μl 5X SDS dye. A 12% SDS gel was used to resolve the proteins. The gel

was scanned to ensure protein loading amounts remained equal after staining and distaining for 5

minutes. After scanning the gel was dried for 2 hours using filter paper and vacuum at 90°C. The dried

gel was then loaded into a phosphor-imaging cassette for 48 hours then scanned on a Typhoon scanner.

A double mutant, R174K/R106K, was tested after single point analysis.
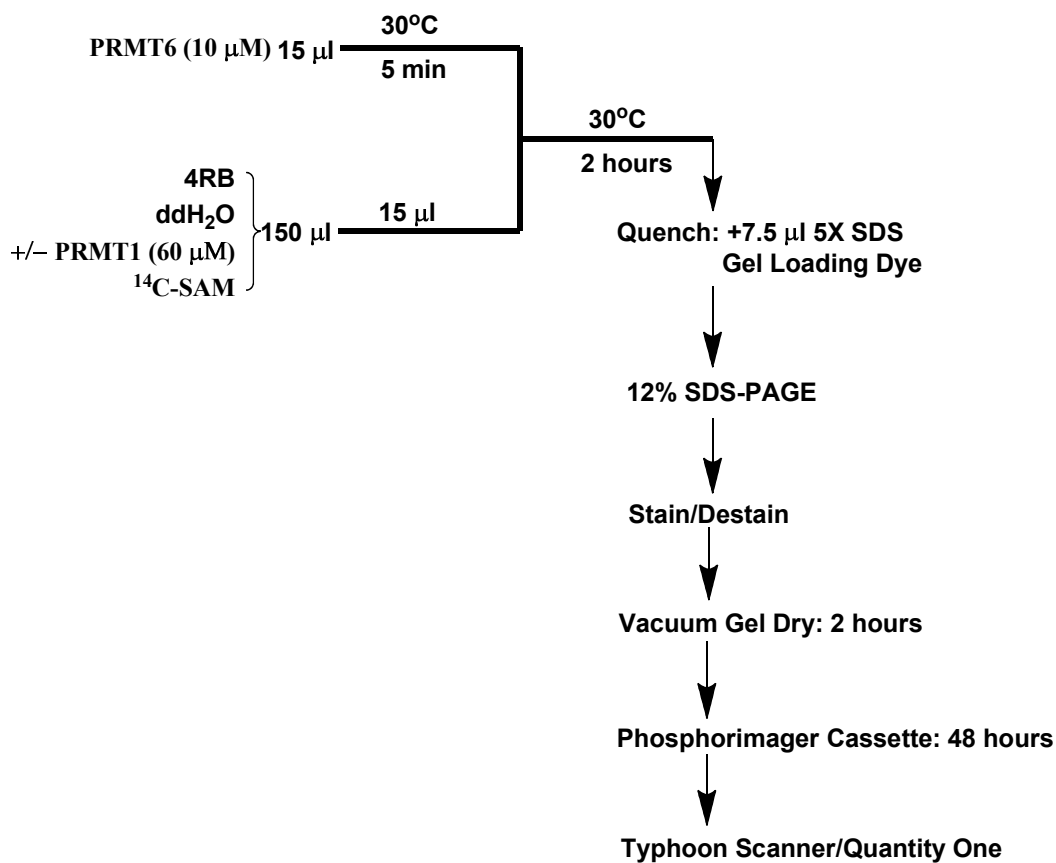
Scheme 2.2- Radioactive Methyltransferase Assay Scheme for PRMT6 Mutants

2.1.8   *Steady state kinetics of PRMT6 mutants R174K, R106K, M60L, and M166A*

The initial kinetic studies for PRMT wt and mutants were detected on a H3(1-31) peptide (sequence: ARTKQTARKSTGGKAPRKQLATKVARKSAPA) . The PRMT6 concentration was 0.6 µM, substrate concentration ranged from 0 to 200 µM. , and ^{14}C-SAM was 30 µM. The reaction buffer used was the same as previously stated. A reaction mixture containing substrate, ^{14}C-SAM, and RB was placed at 30 °C for 5 min. The reaction was initiated with the addition of enzyme. The reaction took place for 60 min and was then quenched by placing on p81 filter paper (Whatman). After initial air drying the p81 paper was washed with 50 mM NaHCO$_3$ (pH 9) and air dried. The p81 paper was placed in scintillation tubes and combined with a scintillation cocktail to measure the methylated product. The double mutant was performed using the same conditions.
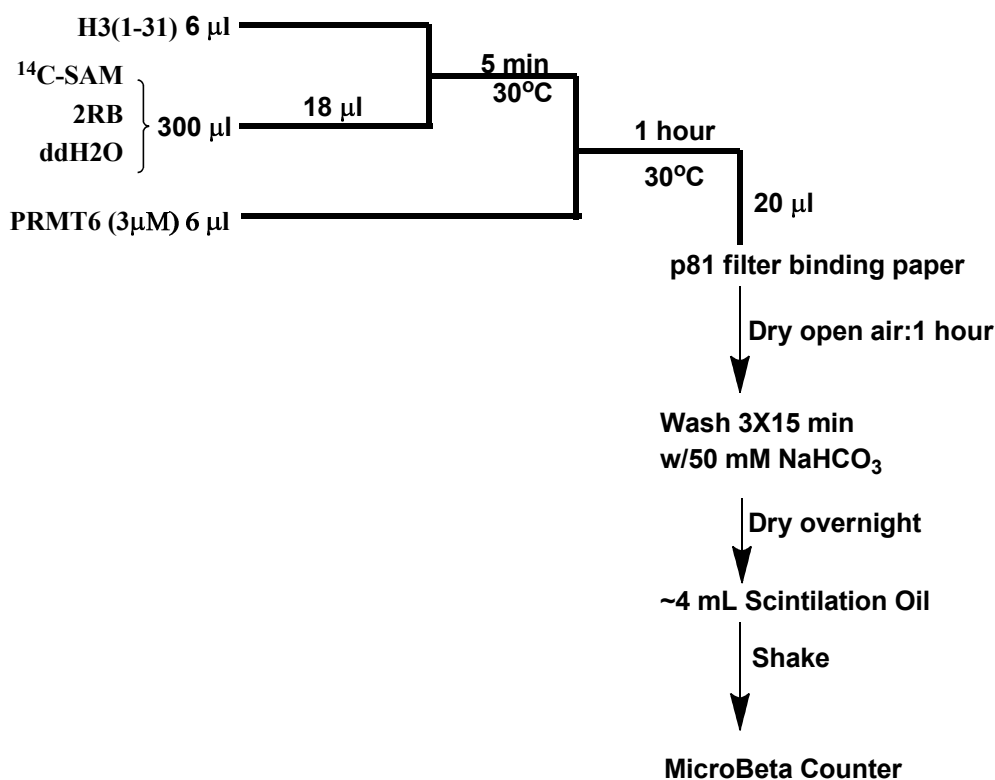
Scheme 2.3- Radiolabelled Methyltransferase Assay for Kinetics

Eqaution 2.3- Micharlis Menten eqaution

2.1.9   GST-, HIS-, and tag-free PRMT1 activity on H4, H4-20, and PRMT6

The activity of different PRMT1 enzymes was detected using H4, H4-20, and PRMT6. $0.4\mu M$ TF-, GST-, and His-PRMT1 was reacted with 10 μM H4/H4(1-20) and 3.7 μM PRMT6. The reaction buffer used is the same of previous experiments. H4 and H4(1-20) was incubated in a reaction mixture of 2RB, ^{14}C -SAM, and ddH$_2$O at 30°C for 5 min prior to the addition of PRMT1 to initiate the reaction. PRMT6 can automethylate and incubation was reversed with PRMT1 to avoid premature methylation. PRMT1 was incubated in a reaction mixture of 2RB, ^{14}C -SAM, and ddH$_2$O at 30°C for 5 min prior to the addition of PRMT6 to initiate the reaction. The reactions were carried out at 30°C for 30 min and quenched with the addition of 5X SDS loading dye. The samples were resolved on a 15% SDS gel using 200V. The gel was dried for 2 hours using filter paper and vacuum at 90°C. The dried gel was then loaded into a phosphor-imaging cassette for 48 hours then scanned on a Typhoon scanner.

2.2   Dimerization Inhibitor Experiments

2.2.1   Synthesis of dimerization inhibitor peptides

Table 2.3 -Dimerization inhibitors peptides compared to the dimerization arm

Name	Sequence	Calculated MW
Dimerization Arm (29)	RQYKDYKIHWWENVYGFDMSCIKDVAIKE	3666.2 g/mol
DMI-22	Ac-KIHWWENVYGFDMSCIKDVAIK-H	2725 g/mol
DMI-26	Ac-KDYKIHWWENVYGFDMSCIKDVAIKE-H	3260.74 g/mol

Solid Phase Peptide Synthesis was performed using the fmoc strategy on a PS3 peptide synthesizer from Protein Technologies Inc. 20% Piperidine/DMF was used for fmoc deprotection. HCTU was used as the coupling reagent in N-methylmorpholine. The resins used were fmoc-Lys(Boc)-wang resin

and fmoc-Glu(OtBu)-wang resin. The resins were weighed down with glass beads through the synthesis. The N terminal was capped using acetic anhydride. After synthesis the resin was washed with DMF and DCM and dried in a lyophilizer. The peptides were then cleaved using 94% TFA, 2.5% H_2O, 2.5% TIS, and 1% EDTA for 5 hours. The peptides were precipitated using cold ethyl ether while centrifuging at 5,000 RPM for 10 min at 4^oC to isolate the peptide. After decanting the supernatant the precipitant was air dried and redissolved in acetonitrille and H_2O. A crude sample was sent for testing for ESI and Maldi MS. Once confirmation that peptide target peptide is present in the crude the peptides were purified on a reverse phase HPLC. Peaks were combined and the acetonitrille was removed using rotary evaporation. The collected samples were re-lyophilized. Peptide dissolved in 20% DMSO in ddH_2O as needed.
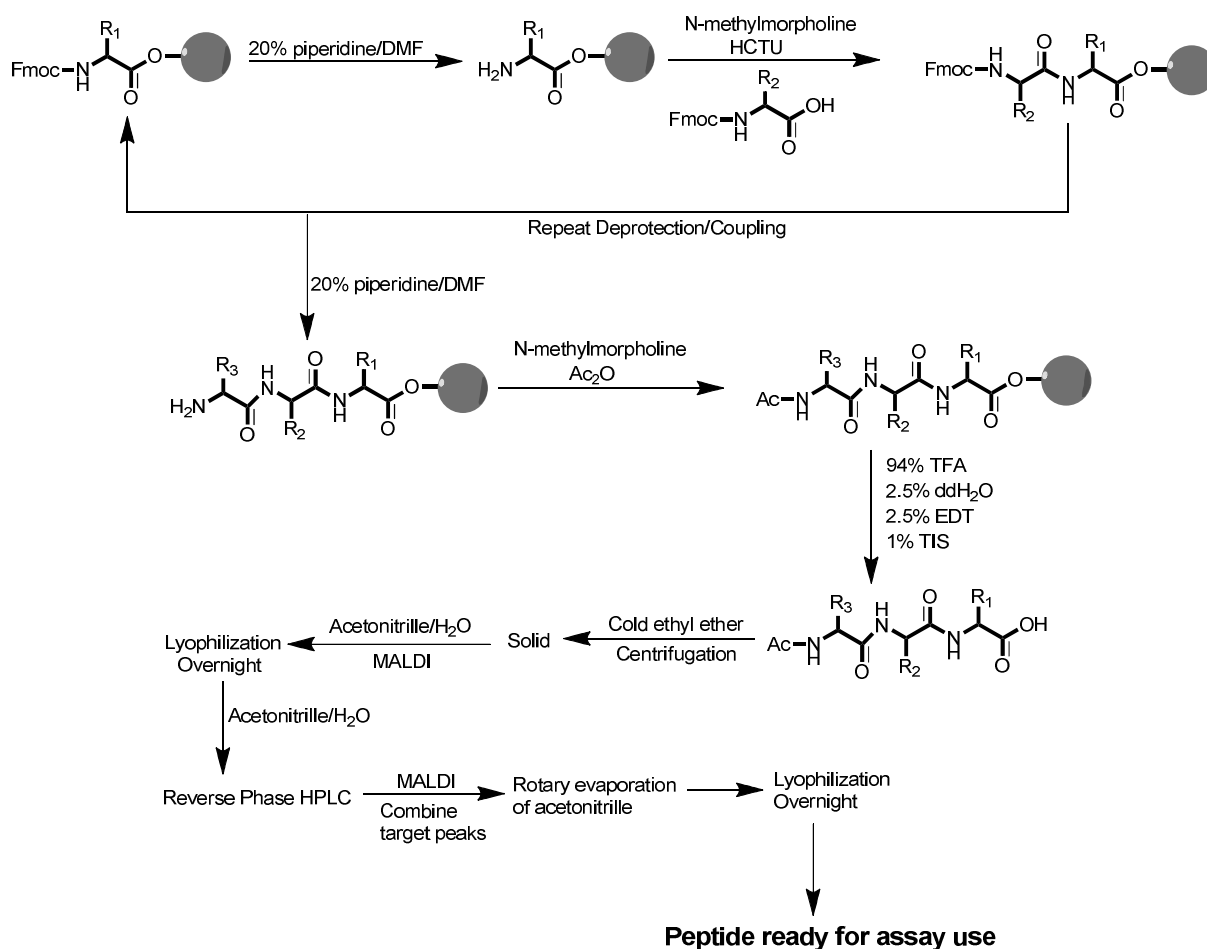
Scheme 2.4- Peptide synthesis experimental procedure

2.2.2   2,2,2-trifluoroethanol effect on PRMT1 activity

TFE was tested at 0%, 5%, 10%, 15%, 20%, 25%, and 30% for the effect of H4 methylation by PRMT1. The final concentrations used were 0.4 μM PRMT1, 5 μM H4 protein, and 3 μM H^3-SAM. TFE was placed in a reaction mixture of H^3-SAM, H4, ddH$_2$O, and reaction buffer for 7.5 minutes at 30°C. PRMT1 was added and let to react for 1 hour before quenching on p81 paper. The p81 paper was washed with 50mM NaHCO$_3$ 3 times for 15 minutes each and then dried in the oven. Scintillation cock-tail was introduced to measure the counts on a MicroBeta2 from PerkinElmer.

2.2.3   Western blot: effect of DMI on oligomeqrization of PRMT1

DMI 22 and DMI 26 were incubated with 1μM PRMT1 in storage buffer (HEPES pH 7 50mM, NaCl 500 mM, EDTA 2mM, Glycerol 20%, DTT 2 mM, and Triton X-100 0.1%) overnight. The peptide con-centrations ranged from 0-131.6 μM. Peptides were dissolved in 20 % DMSO and the addition of 20% DMSO to keep the final % of DMSO constant at 4%. The samples were reacted with 0.15% glyceralde-hyde for 5 minutes at room temperature. The reaction was quenched with 12 μl 5x SDS dye. The sam-ples were then boiled at 95°C for 6 minute then resolved on an 8% SDS gel. Proteins were transferred to the nitrocellulose membrane and blocked with 5% nonfat milk TTBS for 1 hour at room temperature. Several loading amounts of primary antibody (anti-PRMT1 rabbit) were tested for optimization: 1:2500, 1:3000, 1:3333, 1:4000, 1:6000. Incubation with the primary antibody was carried out for 1 hour initially then reduced to 40 minutes. The membrane was washed 3 times for 5 minutes each with 1x TTBS buffer after incubation. Secondary antibody (Anti-Rabit) was then used at different ratios: 1:6250, 1:7500, 1:8333, 1:15000. The same incubation times as primary antibody was used. The membranes were then washed 3 times for 5 minutes each with 1x TTBS buffer. The membrane was then soaked in 1:1 stable peroxide solution and luminal enhancer solution then exposed to the autoradiography film for 30 se-

conds, 1 minute, and 2 minutes and developed on the Kodak X-OMAT. A crude sample of unpurified DMI 22 was used for initial studies prior to the purification of DMI 26.

2.2.4   Time dependent PRMT1 dimerization disruption by DMI 26

DMI 26 and PRMT1 incubation for the Western blot was repeated. 100 μM of DMI 26 was incubated with storage buffer and 1 μM PRMT6 at controlled time points. At each time point the cross linking with 0.15% glyceraldehydes for 5 minutes at room temperature and then quenched with the addition of 5x SDS gel loading dye. The quenched solutions were stored in the -80°C after flash freeze with liquid nitrogen till all time points were collected. The incubation time points tested were 0 (45 sec), 2, 4, 6, 8, 10, and 12 hours. After all time points were collected the samples were heated at 95°C for 6 minutes. The samples were resolved on an 8% SDS gel. The gels were presoaked in transfer buffer before being transferred to the nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TTBS for 1 hour at room temperature. The primary antibody, Anti-PRMT1 from rabbit, was used at 1:6000 in 5% nonfat milk TTBS for 30 min and 40 min at room temperature. The membranes were washed with 1x TTBS 3 times for 5 minutes. Secondary antibody, Anti-Rabbit, was used at 1:15000 in 5% nonfat milk TTBS for 30 min and 40 min. Washing procedure was repeated and then the membrane was then soaked in 1:1 stable peroxide solution and luminal enhancer solution then exposed to the autoradiography film for 30 seconds, 1 minute, and 2 minutes and developed on the Kodak X-OMAT. A 5 min exposure of each was taken as well.

2.2.5   PRMT1 activity with DMI26 at initial dimerization disruption

0.4 μM PRMT1 was incubated with 1RB and [DMI] from 0-50 μM for 4 hours at 4°C. The raction was initiated with the addition of ^{14}C-SAM for a reaction period from 0-120 min at 30°C. The final concentration of ^{14}C-SAM was 10 μM. At each time point the reactions were quenched by loading 20 μl onto p81 paper. After initial air drying the p81 paper was washed with 50 mM NaHCO$_3$ (pH 9) and air

dried. The p81 paper was placed in scintillation tubes and combined with a scintillation cocktail to measure the methylation activity.

2.2.6 IC_{50} test for DMI on PRMT1

The IC50 study for PRMT1 with increasing concentrations of DMI were detected on a H4(1-20) peptide . The PRMT1 concentration was 0.1 μM, substrate concentration was 3 μM, and ^{14}C-SAM was 1 μM. The DMI concentration used ranged from 0 - 127.2 μM. The reaction buffer used was the same as previously stated. A reaction mixture containing substrate, PRMT1, and RB was placed at 4 °C for 4 hours when DMI was added to allow for DMI binding. The reaction was initiated with the addition of ^{14}C-SAM. The reaction took place for 20 min at 30°C and was then quenched by placing on p81 filter paper (Whatman). After initial air drying the p81 paper was washed with 50 mM $NaHCO_3$ (pH 9) and air dried. The p81 paper was placed in scintillation tubes and combined with a scintillation cocktail to measure the methylation activity.

2.2.7   FPLC for visualizing oligomeric states of PRMT1

Samples of control and monomer PRMT1 were prepared by incubating PRMT with/without DMI for 4 hours at 4°C. 20% DMSO was added to the control to keep DMSO constant for each sample. 500 μl samples were prepared with 100 μM DMI and 20 μM PRMT1 for injection into the AKTApurifier FPLC using a 100 μl loop. FPLC running buffer was prepared and filtered on ice prior to loading onto the instrument. The FPLC was allowed to equilibrate overnight with 300 ml of FPLC running buffer with a flow rate of 0.5 ml/min at 1.0 psi. The next day the injection loop was washed 3 times with FPLC running buffer and then the sample was loaded to the loop. The FPLC was run with a flow rate of 1 ml/min at 1.12 psi for 6 hours. The chromatograms were overlaid to check the retention times of the monomer and oloigomer.

2.2.8   Native PAGE-Western blot of PRMT1 and DMI

Native gels were prepared similar to the gels used prior. SDS was removed from the gel recipe, sample loading buffer, and running buffer. Samples containing 1 μM PRMT1 with/without 100 μM DMI or 20% DMSO and 5 μM SAM were incubated for 4 hours. One sample was prepared with PRMT1, SAM, and DMI during the 4 hour incubation. Another sample was designed to emulate the kinetic assays where SAM is added for 5 min incubation after the initial 4 hour incubation. The other two prepared were PRMT1 with DMSO and PRMT1 with DMI. The samples were resolved on an10% native gel for 4.5 hours at 100V. The gels were presoaked in transfer buffer before being transferred to the nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TTBS for 1 hour at room temperature. The primary antibody, Anti-PRMT1 from rabbit, was used at 1:6000 in 5% nonfat milk TTBS for 30 min and 40 min at room temperature. The membranes were washed with 1x TTBS 3 times for 5 minutes. Secondary antibody, Anti-Rabbit, was used at 1:15000 in 5% nonfat milk TTBS for 30 min and 40 min. Washing procedure was repeated and then the membrane was then soaked in 1:1 stable peroxide solution and luminal enhancer solution then exposed to the autoradiography film for 30 seconds, 1 minute, and 2 minutes and developed on the Kodak X-OMAT.

2.2.9   Steady state kinetics of PRMT1 with/without DMI

The kinetic study for rPRMT1 was performed with 100 μM DMI at varying H4(1-20) peptide concentrations. The PRMT1 concentration was 0.04 μM, substrate concentration was 0-20 μM, and ^{3}H-SAM was 1 μM.   The reaction buffer used was the same as previously stated. A reaction mixture containing substrate, PRMT1, and RB was placed at 4 °C for 4 hours when DMI was added to allow for DMI binding. The reaction was initiated with the addition of ^{3}H-SAM. The reaction took place for 15 min at 30°C and was then quenched by placing on p81 filter paper (Whatman). After initial air drying the p81 paper was washed with 50 mM $NaHCO_3$ (pH 9) and air dried. The p81 paper was placed in scintillation tubes and combined with a scintillation cocktail to measure the methylation activity.

H4(1-20) 6 μl

DMI
PRMT1
2RB
ddH2O
} 390 μl

22.4 μl

4 hours
4°C

5 min
30°C

1 hour
30°C

³H-SAM 1.6 μl

20 μl

p81 filter binding paper

Dry open air:1 hour

Wash 3X15 min
w/50 mM NaHCO₃

Dry overnight

~4 mL Scintilation Oil

Shake

MicroBeta Counter
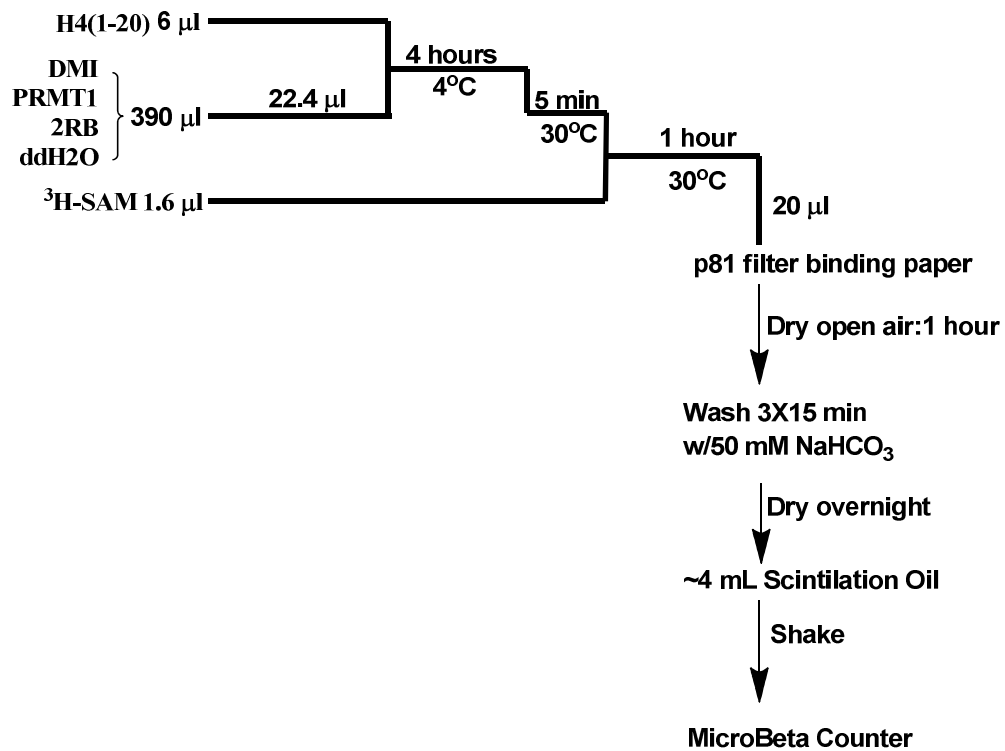
Scheme 2.5- Methyltransferase assay for DMI on PRMT1

3   RESULTS

3.1   PRMT1 Interacts with and Methylates PRMT6

3.1.1   Expression of recombinant rPRMT1was successful

Rat PRMT1 gene was inserted in the pET28a vector. Recombinant PRMT1 (41 KDa) was ex-

pressed in E coli and purified using a Ni affinity column. The protein was resolved on a 12% SDS-PAGE gel

to confirm PRMT1 status throughout the purification (Figure 3.1). The gel was stained with Coomassie

Brilliant Blue for overnight and then destained till the background dye disappeared.
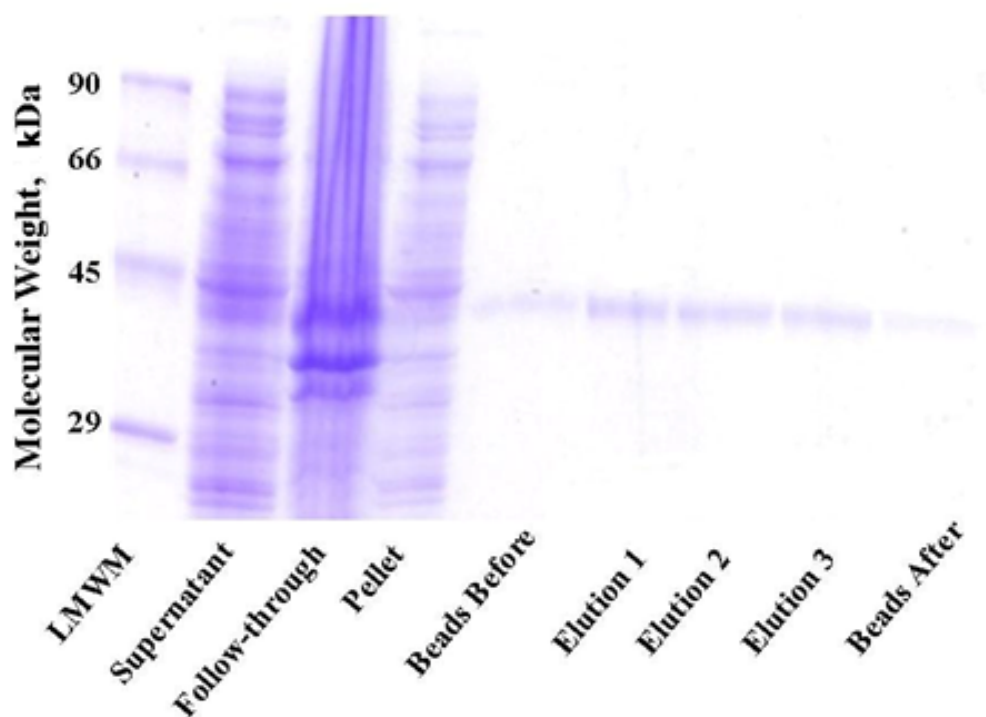
Figure 3.1- 12% SDS-PAGE of PRMT1. The supernatant and pellet was collected after lysis and centrifugation of the E coli. The flow-through was a sample of the elluant after incubation with the Ni beads and supernatant. Elution fractions were collected to track protein elution every 10 mL. A small sample of Ni beads were collected and resolved to track protein binding. There are some PRMT1 left on the beads after elution. Slower/longer elution periods could solve this. The protein amount obtained was 2.46 mg/ml (60 μM). Purity of PRMT1 is sufficient for assays.

3.1.2   PRMT6 mutants were successfully obtained from site directed mutagenesis

To investigate the methylation site(s) of PRMT6 by PRMT1, we carried out site directed muta-

genesis. The gene of PRMT6 was in pET28a vector. Mutation of R174, R106, and R29 were repeated mul-

tiple times to obtain proper banding and mutation. Bad mixing and improper handling of the reaction

mixture in initial experiments could have caused these errors (Figure 3.2). The light bands were chosen

for Dpn1 digestion prior to XL1-Blue transformation. Obtaining colonies with the correct sequence

proved difficult with R29K and R106K. Several colonies were chosen for DNA extraction to ensure that

the mutations were intact for protein expression. The DNA sequences obtained were analyzed using

NCBI Blast alignment program to determine if the mutation was successful. Sequence matches for the

full sequence from T-7 and T-7 terminator were 100% (Appendix).
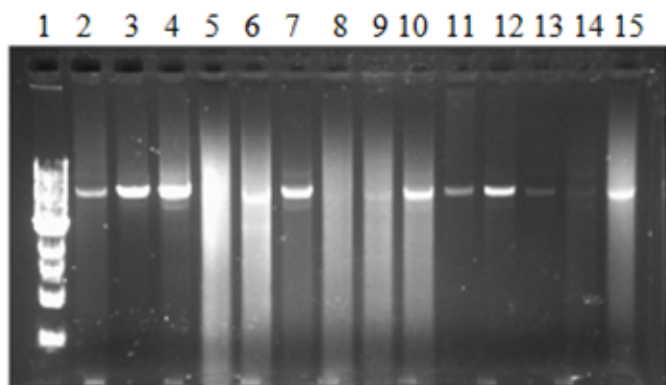
Figure 3.2- 1% Agarose gel electrophoresis of the PCR reaction mixtures. 1% agarose gel after imaging. 1. DNA ladder, 2-4. R29K, 5-7. R82K, 8-10. R106K, 11-13. R228K, 14-15. R174K. Wells loaded with samples of increasing DNA template shown in Table 2.1. Lanes with dim bands taken for Dpn1 digestion.

3.1.3   Expression and purification of PRMT6 wt and mutants successful

Rat PRMT6 gene was inserted in the pET28a vector. Recombinant PRMT6 (41.9 KDa) was ex-

pressed in E coli and purified using a Ni affinity column.   All protein fractions during the purification

process were monitored by SDS-PAGE (Figures 3.3-3.11). Gels loading consisted of LMWM, supernatant,

pellet, flow through, beads before, elution 1, elution 2, elution 3, beads after, and concentrated protein

unless stated otherwise. The supernatant and pellet collected after centrifugation of lysed E coli. The

flow through was collected after supernatant incubation with the beads. Elution samples were collected

for the first 3 elution fractions with 10 ml elution buffer. A small sample of the Ni beads before and after

elution was tested for protein binding and elution.
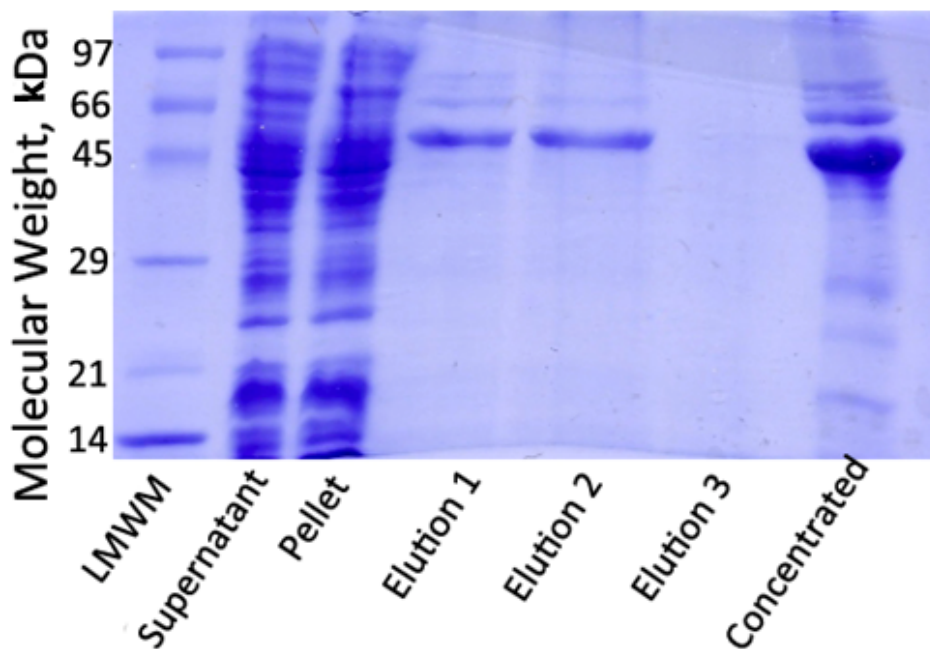
Figure 3.3- 12% SDS PAGE of PRMT6-R82K. 8 wells were used due to poor well conditions on the ends. PRMT6-R82K mutant expressed very well. The concentration obtained was 1.510 mg/ml (35.9 μM) with a final volume of 2 ml.
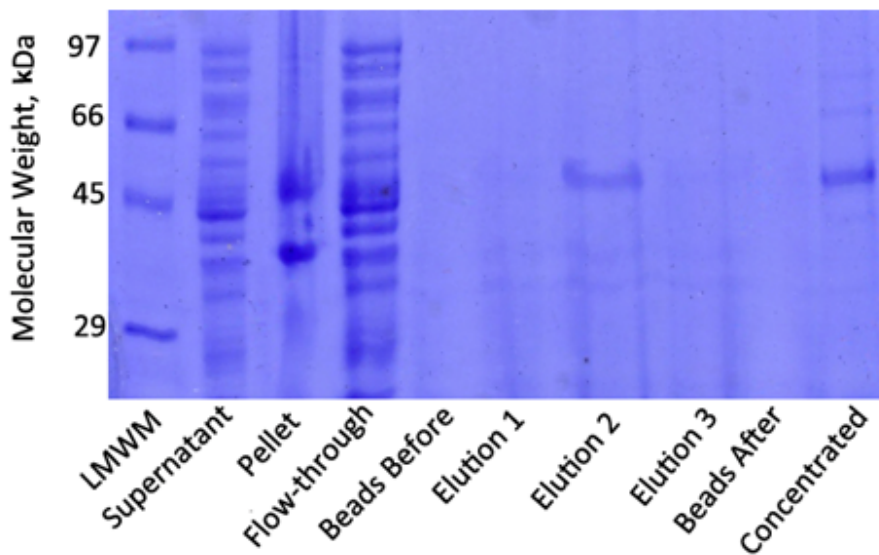
Figure 3.4- 12% SDS PAGE of PRMT6-R228K. Banding for the concentrated protein was observed; however, the first concentration measured was 0.1697 mg/ml (4.04 µM). The protein was further concentrated to 100 µl and the concentration measured was 1.755 mg/ml (41.8 µM)
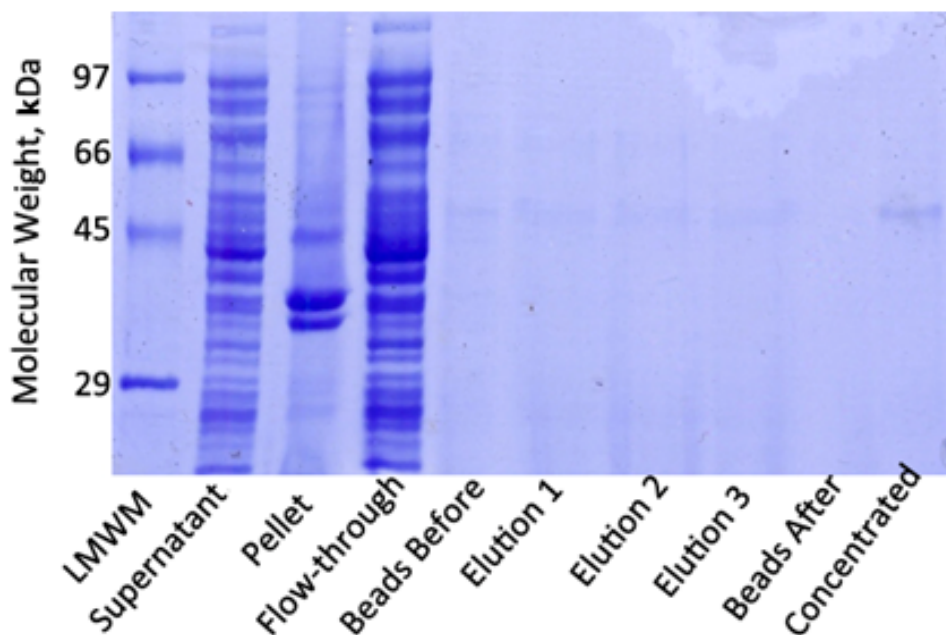
Figure 3.5- 12% SDS PAGE of PRMT6-R174K. R174K proved very difficult to express. This mutant was expressed and purified 3 times before the actual protein was obtained. This site may be important to the structure causing the protein to be unstable. The first purification yielded no protein. The second purification yielded a degraded protein. The third exhibited proper protein bands. The concentration measured was 1.45 mg/ml (34.5 µM) with a final volume of 200 µl.
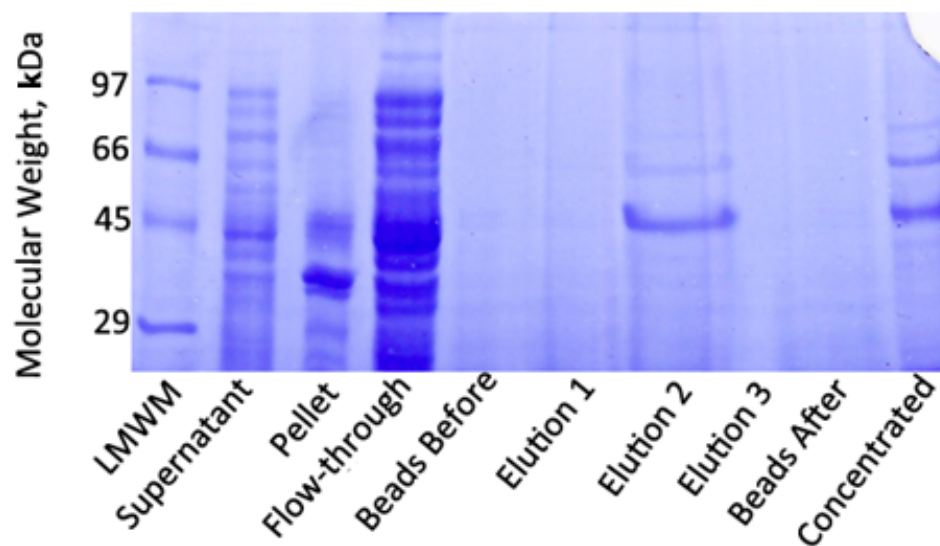
Figure 3.6- 12% SDS PAGE of PRMT6-M166A. M166A exhibited low expression levels. The initial concentration measured at 2 ml was 8.068 μM. The volume was concentrated 110 μl and the concentration measured was 2.193 mg/ml (52.2 μM)
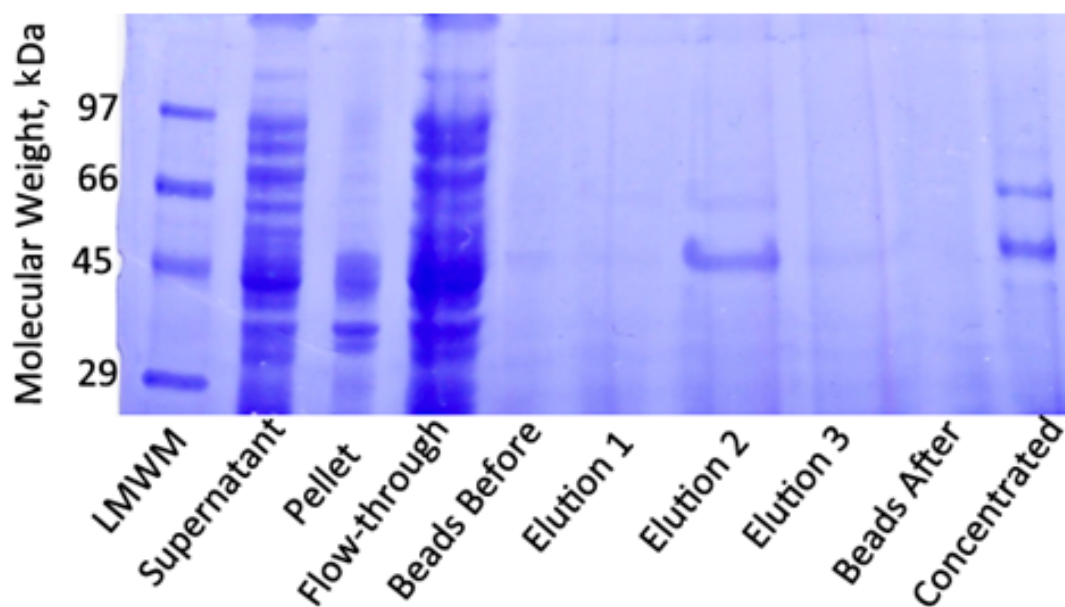
Figure 3.7 12% SDS PAGE of PRMT6-R29K. R29K exhibited low expression levels. The first concentration measured was 5 μM at 500 μl. The volume was further concentrated to 90 μl and the concentration measured was 0.9839 mg/ml (23.4 μM).
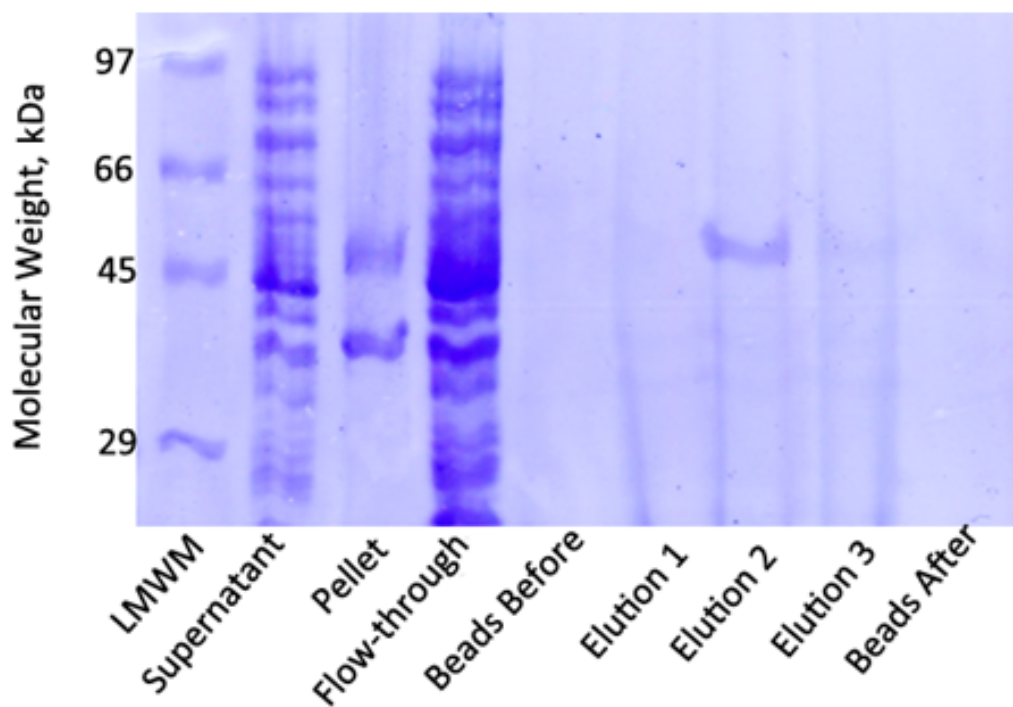
Figure 3.8- 12% SDS PAGE of PRMT6-R106K. PRMT6-R106K mutant was difficult to express. The mutant was expressed and purified 3 different times until even small banding was observed in the SDS gel. The gel above is from the second purification. Banding was observed in elution 2. Repeated trials show that the expression is very small and probably unstable. This too could be because of the importance of this site to the structure. Dr. You Feng purified the third trial and the final concentration was measured to be 0.4782 mg/ml (11.38 μM) with a final volume of 200 μl.
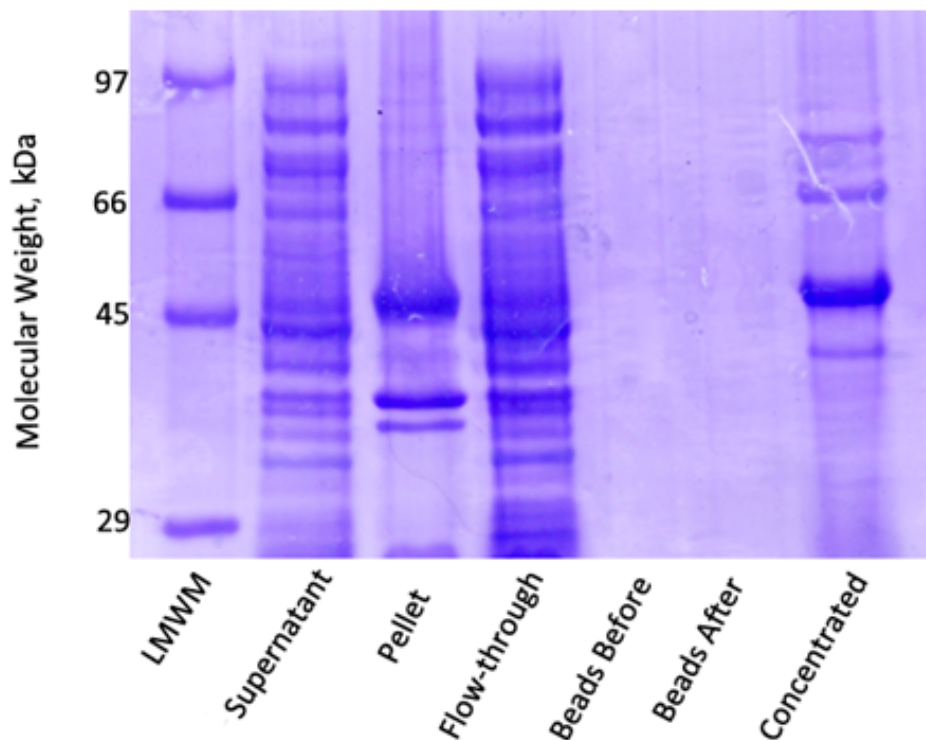
Figure 3.9- 12% SDS-PAGE of PRMT6-M60L.The elution fractions were not collected during puri-
fication. PRMT6-M60L mutant exhibited good expression levels. The concentration measured
was 0.86 mg/ml (20.6 μM) at ~900 μl.
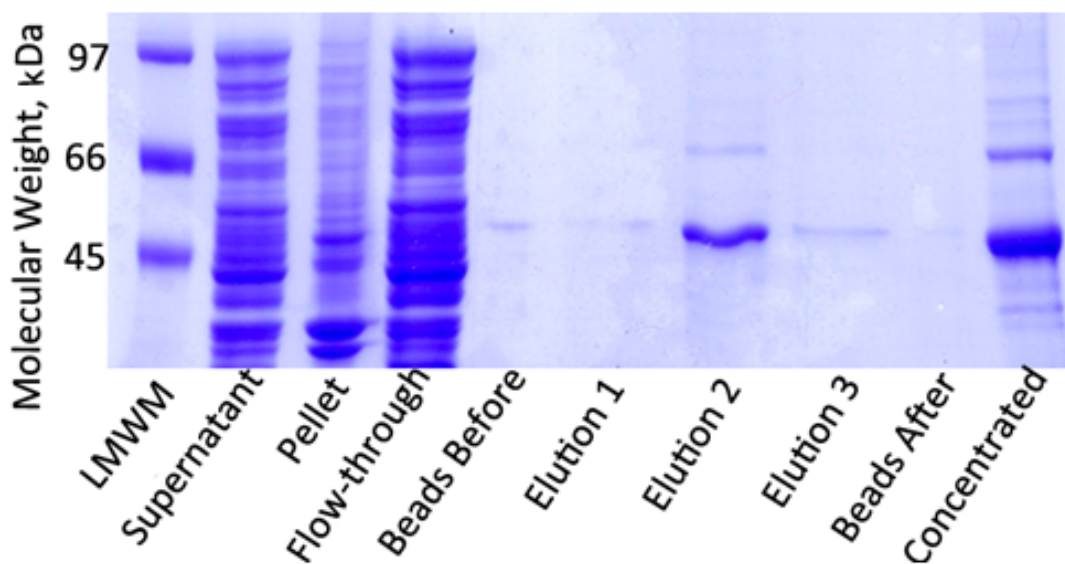
Figure 3.10- 12% SDS PAGE of wt-PRMT6. Wt-PRMT6 showed really good expression levels. The
concentrated protein well shows similar impurities observed in all the mutant gelsl. The concen-
tration measured was 1.168 mg/ml (27.8 μM). Re-expression of wt-PRMT1 22 μM and 18.3 μM
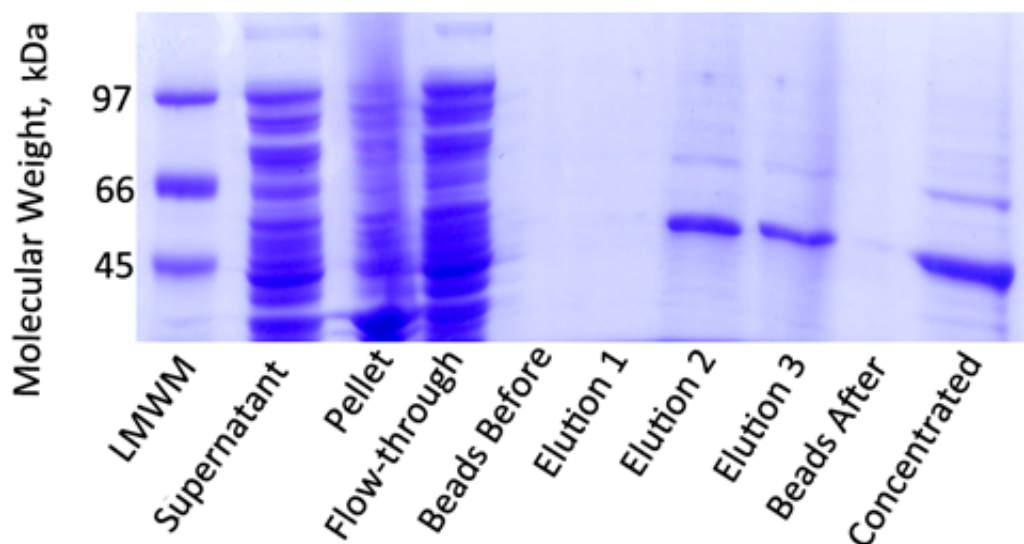
Figure 3.11- 12% SDS PAGE of PRMT6-R106K/R174K. The double mutant showed good expression levels with colonies on all plates. It was not as difficult as each of the single point mutations. The final concentration measured was 0.56 mg/ml (13.4 μM). Re-expression showed a concentration of 12.4 μM.

For most of the proteins, the expression levels were acceptable after one expression. The expression of PRMT6 -R106K and -R174K mutants were performed multiple times due to low expression levels. Concentrations of PRMT6 mutants were isolated with acceptable concentrations ≥ 10 μM at low volumes from 1mL-90 μl. Similar impurities were observed in each gel for each mutant.

3.1.4   PRMT1 methylates PRMT6

Interestingly, we found that PRMT6 interacts with PRMT1. The purpose of the experiment was to show that PRMT1 can methylate PRMT6. Because previously it has been shown that PRMT6 has the ability to automethylate [32], it is an important question to demonstrate that whether PRMT6 automethylation is as strong as PRMT1 mediated methylation. Radiometric assays for PRMT6 in the presence of ^{14}C-SAM with/without H3.1 protein, PRMT1, or PRMT1-E153Q were performed to observe the methylation of PRMT6 (Figures 3.11-12). The experiment was performed for 3 times to assure con-

sistency in methylation patterns. Inactive PRMT1-E153Q was added to demonstrate the case that only

PRMT6 automethylation occurred. H3 protein was added to test substrate methylation. PRMT1 was

tested without PRMT6 as a control.
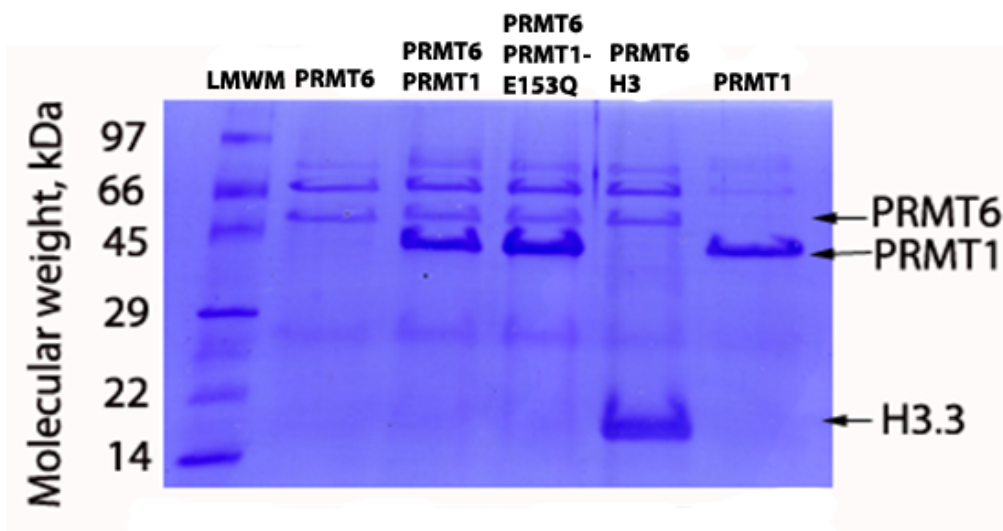
Figure 3.12- Commasie blue staining of 12% SDS-PAGE of PRMT1-PRMT6 interactions. The lanes were labeled in the figure. Proper separation of PRMT6 and PRMT1 can be observed on the SDS gel. The loading amounts for PRMT6 and loading amounts of RMT1 and PRMT-E153Q appear to be equal in the gel.
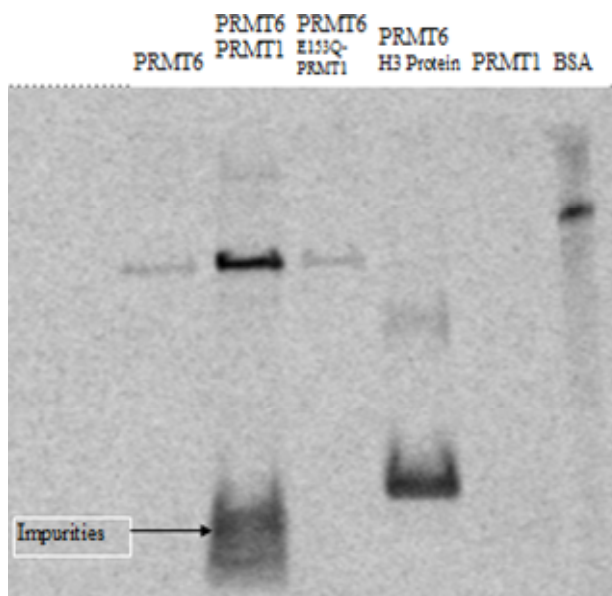
Figure 3.13- Phosphorimage of PRMT interaction. The image is labeled above as previously labeled on the gel. Impurities are present in the PRMT6/PRMT1 and PRMT6/H3.3 lanes after

phosphorimage storage. The impurities in the phosphorimage not present in the SDS gel in Figure 3.12. The impurity in the PRMT1/PRMT6 lane may be $^{14}CH_3OH$ from hydrolyzed ^{14}C-SAM. PRMT6/H3.3 lane shows slight impurities near the PRMT6 band.

As seen in Figure 3.13, the results show that PRMT1 strongly methylates PRMT6, and PRMT6 automethylation without PRMT1 is significantly weaker. The inactive mutant PRMT1-E153Q shows a return to PRMT6 automethylation levels. This can possibly rule out that PRMT1 is increasing PRMT6 automethylation by inducing a conformational change. In the presence of the H3 protein substrate, PRMT6 did not automethylate and preferentially methylated the H3.3. PRMT1 was loaded as a control, showing lack of automethylation in PRMT1. The impurities shown in the phosphorimages are not visible in the SDS gel suggesting the impurities may be from hydrolyzed SAM or degraded peptide fragments.

3.1.5   *PRMT6 automethylation is slower than PRMT1 mediated methylation*

The methylation rate analyses of PRMT6 with/without PRMT1 or PRMT1-E153Q were designed to compare the amount of methylated product formation for each condition. A reaction mixture containing RB, ddH$_2$O, and ^{14}C-SAM, with/without PRMT1 or PRMT1-E153Q, was reacted with PRMT6 and quenched at different time points over a span of 2 hours. The samples collected at each time point were analyzed on the SDS gel by Coomassie stain and phosphorimage (Figure 3.14). PRMT1 mediated methylation of PRMT6 showed more intensity than that of the PRMT6 by itself and PRMT6 with inactive PRMT1-E153Q. The PRMT1-mediated methylation of PRMT6 was measured by taking the total observed methylation and subtracting out the possible automethylation attribution. The quantified data was plotted to compare the overall differences between each strategy (Figure 3.15). The initial rates were obtained from 0-30 min. PRMT1 mediated methylation is 0.0078 ± 0.0028 µM/min and the automethylation is 0.0028 ± 0.0015 µM/min which is a 3 fold difference. There is a 4 fold difference from the total methylation rate (0.0106 ± 0.0041 µM/min) from the automethylation rate. PRMT6 automethylation in the presence of PRMT1-E153Q is 0.0015 ± 0.012 µM/min, which is 2 fold smaller

that PRMT6 automethylation rates without PRMT1-E153Q. PRMT6 automethylation in the presence of

PRMT1-E153Q shows a slight decreased rate which may be due to interaction of SAM with the inactive

mutant. PRMT1-E153Q has been shown to not be able to stabilize Arg in the active site, but SAM binding

is not affected[29].
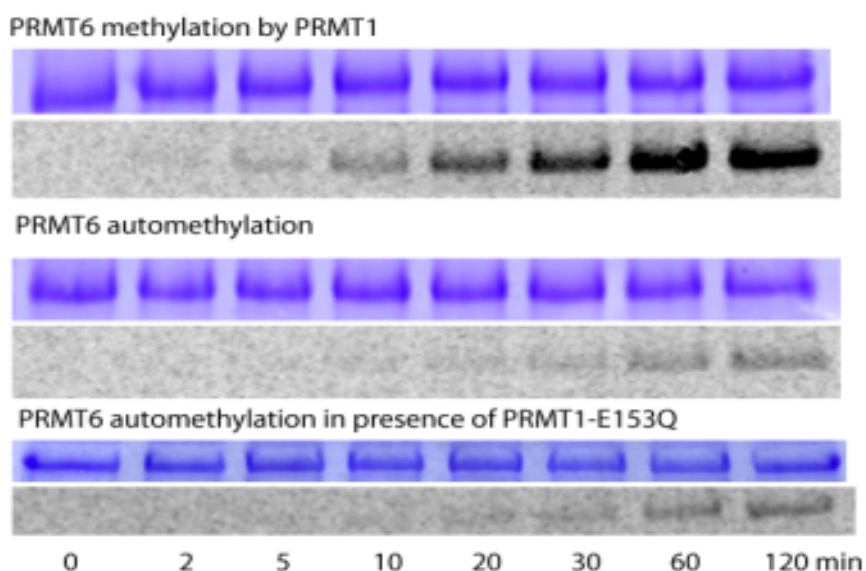
Figure 3.14- Coomassie Blue stain of SDS gel and phosphorimage. The PRMT6 band in the SDS
shows equal loading. Methylation was tracked for PRMT6 over a 2 hour time period
with/without PRMt1 or PRMT1-E153Q. The initial time point was place into SDS loading dye pri-
or to mixing. 30 μl was taken out for each time point from the reaction mixture and quenched
with SDS loading dye. The gels were visualized with Coomassie staining then dried for phosphor
storage for 48 hours to visualize product formation.

Gel loading on the Coomassie Blue staining shows an equal amount for each lane and the

phosphorimage shows an increase in intensity as time increases. PRMT6 automethylation with/without

PRMT1-E153Q showed similar intensities. Automethylation was only distinguishable at 20 min of the

reaction. The PRMT6 methylation band is more prominent when PRMT1 is in the reaction solutions.
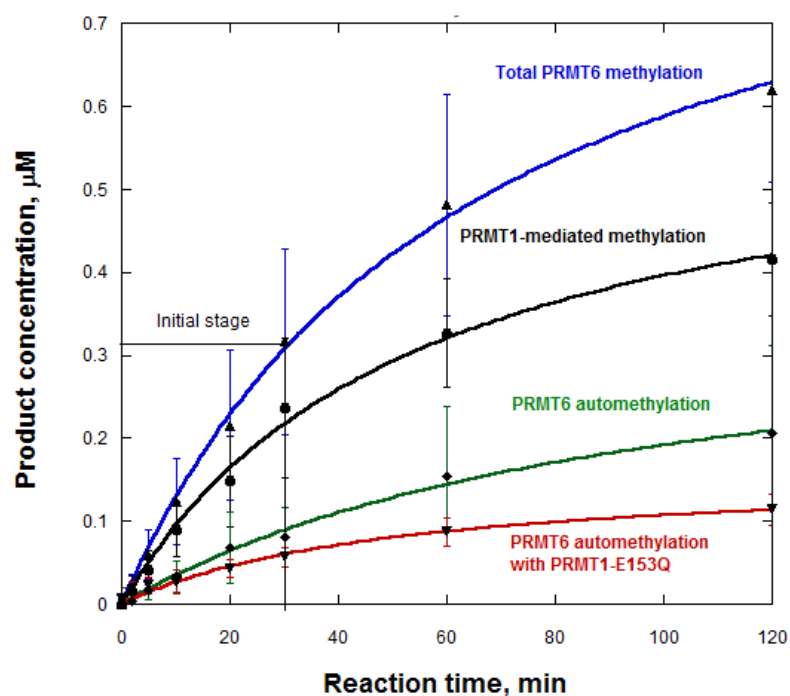
Figure 3.15- PRMT6 time-course methylation. Total PRMT6 methylation includes both PRMT1 mediated and PRMT6 automethylation. PRMT6 auto methylation was subtracted from the total PRMT6 methylation to show PRMT1 mediated methylation of PRMT6. PRMT6 automethylation with PRMT1-E153Q was lower than PRMT6 automethylation. Steady state was achieved between 0-30 min of the reaction.

Initial stages are labeled in the plot. Initial rates were obtained by using the linear slope of the

initial stages. Data analysis of PRMT6 methylation clearly demonstrates the effect of PRMT1 on PRMT6

methylation. In the presence of PRMT1 and SAM the methylation levels significantly increase revealing

that PRMT6 is a novel substrate for PRMT1. In the presence of PRMT1-E153Q we observe a return to

like-automethylation levels showing the significance of the PRMT1 interaction and supporting that

PRMT1 is not causing PRMT6 to automethylate more efficiently. The initial rate showed

automethylation was 3 fold less than that of the PRMT1 mediated methylation. Future research is need-

ed to investigate whether and howPRMT1 methylates PRMT6 in vivo to regulate its function. Combined

with automethylation, PRMT1 mediated methylation could both cross communicate to affect the overall activity of PRMT6 on its biological substrates.

3.1.6   *Methylation of PRMT6 mutants reveal key methylation residues*

Methylation of PRMT6 mutants was analyzed with/without PRMT1 to determine if the mutants are important for either automethylation or PRMT1 mediated methylation. The methylation assays for wt-PRMT6 and each PRMT6 mutant with/without PRMT1 were repeated for three times with adjusted gel loading amounts and to ensure repeatability (Figure 3.16). Gel loading of some mutants varied from preparation and had to be corrected as more experiments were performed. To adjust methylation levels to that of gel loading a ratio was taken between the Coomassie stain and the phosphorimage. The mutants were then compared to the wt-PRMT6 (Figure 3.17).
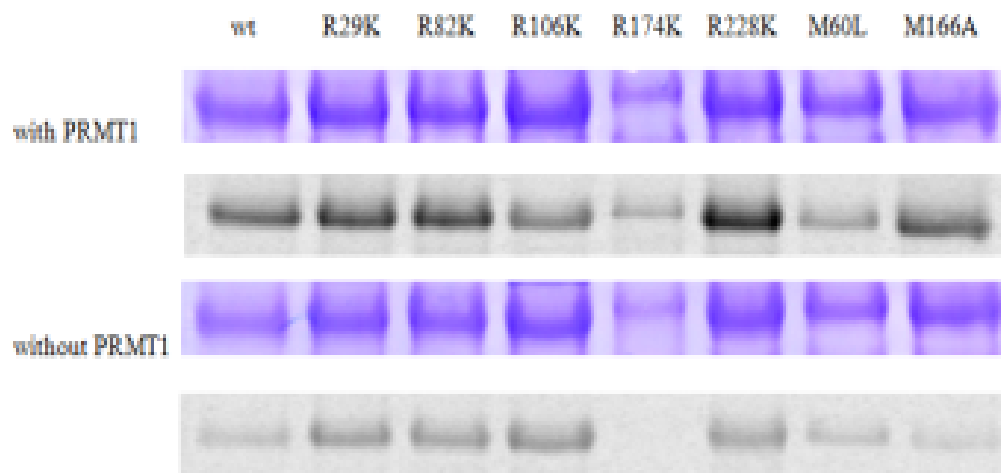
Figure 3.16- Coomassie stain and phosphorimage of PRMT6 wt and mutants. 5 μM PRMT6 and mutants were reacted with/without 1 μM PRMT1 for 2 hours. Gel loading is not equal for each protein and adjustments were made with every repeat.
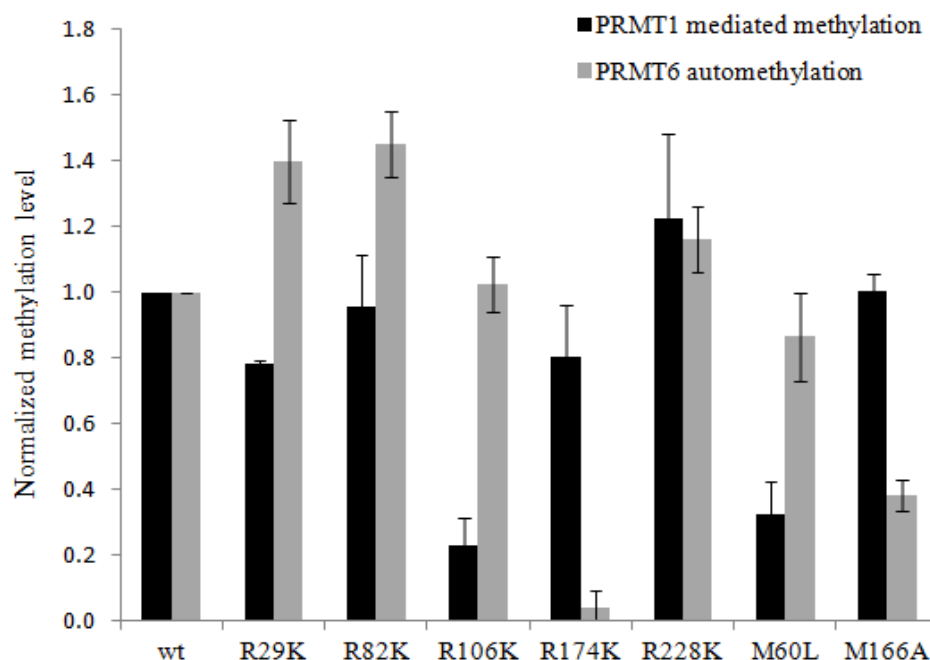
Figure 3.17- Normalized methylation levels of PRMT6 wt and mutants. Methylation levels were normalized to wt-PRMT6. PRMT1 methylation adjusted to compensate for PRMT6 automethylation involvement in the total methylation.

Methylation levels were normalized for wt-PRMT1. The methylation of the R29K and R82K showed increased automethylation but decreased PRMT1 methylation. R228K showed a slight increase for both methylation strategies. R106K showed much less methylation by PRMT1 when corrected for gel loading. Thus, a key residue for PRMT1 mediated methylation could be R106K which retained 33% methylation. R106 lies on the surface of the AdoMet binding domain which can easily be accessed for PRMT1 (Appendix: Figure SI-3). For automethylation, R174K showed only 4% of methylation. The R174 residue is in the AdoMet binding domain near the active site for SAM binding. Perhaps automethylation can occur at this residue in the absence of other substrates due to the close vicinity of the residue to the active site (Appendix: Figure SI-3). R174 is a conserved residue between human and rat PRMT6 variants and is conserved in PRMT1 and PRMT2 [32]. It is possible that this residue is very important for the enzyme and mutation of this residue can be fatal to enzymatic activity. PRMT6-R174K can still be used as a

substrate by PRMT1. The two methionine residues were mutated based on their conservation across the PRMT family. The two mutants correspond to the location of M48 and M155 on PRMT1 which are located in the active site where the substrate arginine is bound. The mutation of these two residues has been reported to alter substrate binding and activity of PRMT1 and give PRMT1 the ability to automethylate and alter substrate binding [33]. M60L showed 33% methylation by PRMT1. M166A showed 38% methylation for automethylation. This shows that the two methionines are possibly important for their related catalysis. Based on the unpublished crystal structure, the two methionines will be in direct contact with SAM and mutation of these residues could affect the binding of SAM causing SAM orientation to shift.
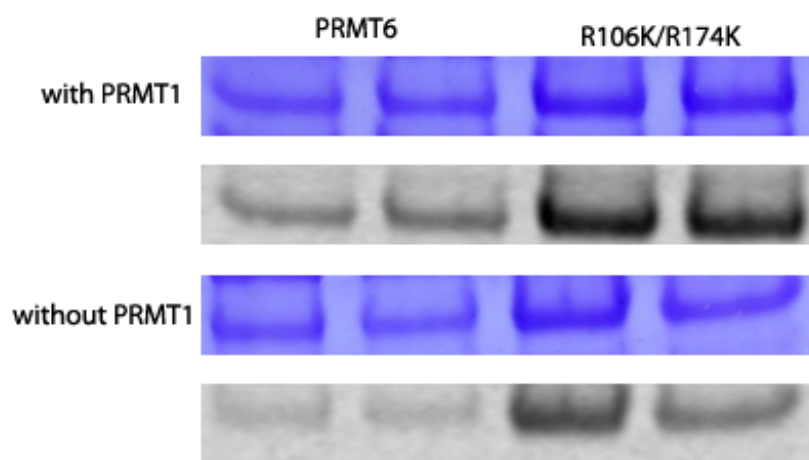
Figure 3.18- Coomassie stain and phosphorimage of PRMT6 wt and PRMT6-R106K/R174K. 5 μM PRMT6 and PRMT6-R106K/R174K were reacted with/without 1 μM PRMT1 for 2 hours. Gel loading was not equal for each protein and adjustments were made to quantify the data.

A double mutant of both key targets was used to determine the effect of the methylation strategies. PRMT6 and PRMT6-R106K/R174K were incubated with/without PRMT1 for 2 hours. The samples were quenched with SDS loading dye and visualized with Coomassie staining and phosphorimaging. The gel once again was not equal loaded and corrections were made to adjust the values to the loading amounts. Banding in the phosphorimage is darker than the wt-PRMT6 in both cases..
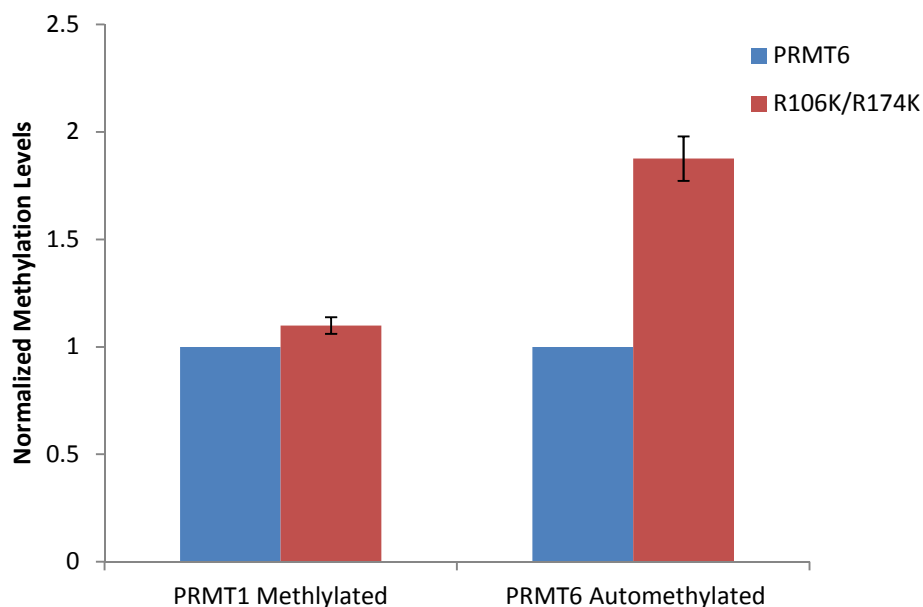
Figure 3.19- Normalized methylation levels of PRMT6 wt and PRMT6-R106K/R174K. Methylation levels were normalized to wt-PRMT6. Quantification was adjusted for gel loading. PRTMT mediated methylation was obtained after the adjustment for PRMT6 automethylation was made.

The data was analyzed using the commassie staining for loading normalization. PRMT6 automethylation was removed from the PRMT1 mediated methylation. From the Figure 3.19, we see a return to wt-PRMT6 methylation by PRMT1. Automethylation is nearly doubled.

Together, two mutants were identified to be important residues for methylation. PRMT1 mediated methylation was lowered for R106K. Thus R106 was confirmed to be an important target for PRMT1. Automethylation was almost completely lost in R174K, which confirmed that R174 was an important target in PRMT6 automethylation. Like all mutational studies we cannot omit that mutation could disrupt protein folding and cause a shift in protein-protein interactions and compromise activity. Further investigation would likely be needed to test the effect of PRMT1 methylation and automethylation on PRMT6. For mutants M60L and M166A we observe a loss in methylation for each category. This could be due to the relative closeness to the major targets as well as their position in the active site. The

R106K/R174K double mutant of both major targets showed nearly double automethylation than the wt

and wt levels of PRRMT1 based methylation. It is difficult to explain the reason at this stage. Perhaps the

lack of the major methylation targets allow for other major methylation sites to shift. It may also be pos-

sible that double mutation causes a distinct structural change for PRMT6 that changes the

automethylation interactions. Since there was not a published crystal structure at the time, the se-

quence of PRMT6 overlaid into the crystal structure of PRMT1 to get a grasp on the location of the mu-

tants (Appendix: Figure SI-3A). More recently a crystal structure of PRMT6 was reported, but not pub-

lished (Appendix: Figure SI-3B). Both residues of the double mutant lie on a α helix in the AdoMet bind-

ing domain and a β sheet on the opposite side. Disruption of R106 and R174 could shift overall methyla-

tion to other Arg residues.

3.1.7   PRMT6 mutants show different catalytic activities in H3(1-31) methylation

PRMT6 was demonstrated to methylate H3. The mutants that had significant loss in methylation

were tested using H3 (1-31) to determine the effect of methylation loss on a known substrate. The H3

methylation data of PRMT6 wt and mutants were fitted to the Michaelis Mentin formula to obtain kinet-

ic parameters in Figure 17. The experimental procedure was performed with You Feng. The kinetic pa-

rameters are summarized in Table 3.1. Wt-PRMT6 had a k_{cat} of 0.0063 ± 0.0006 min^{-1} and a K_m of 8.4 ±

0.7 µM. The observed turnover for R106K was ~2 fold more than the wt (k_{cat} = 0.0122 ± 0.0004 min^{-1}).

R174K showed no detectable activity for the substrate. From the data obtained, R174 automethylation

could be necessary for PRMT6 activity. R106 methylation by PRMT1 could act to suppress PRMT6 activi-

ty. M60L and M166A also show significantly lowered activity. R106K/R174K was ~3 fold more than the

wt (k_{cat} = 0.0188 ± 0.0005 min^{-1}).
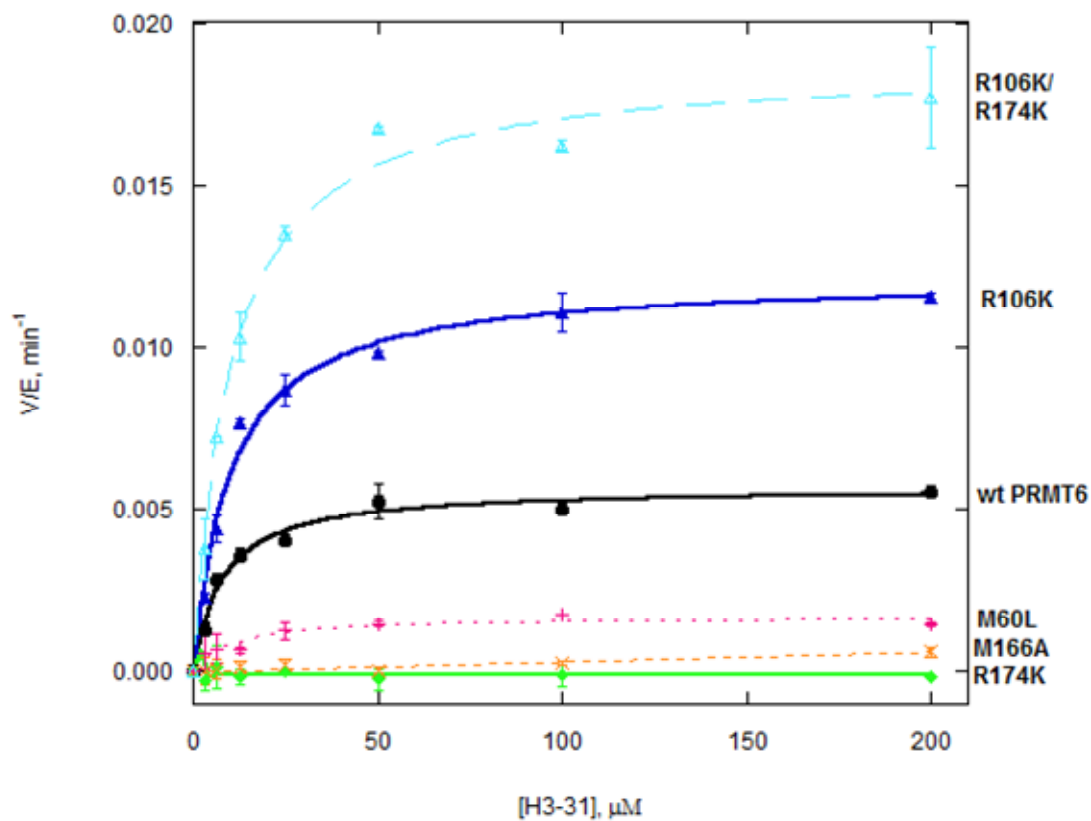
Figure 3.20- Michaelis Menten plot of PRMT6 mutants. The data were fitted to the Michaelis Menten equation. Data for R174K was not able to be fit. M166A curve is hard to observe but was estimated using the program.

Table 3.1 Summarized kinetic parameters

PRMT6 mutant	k_{cat} (min^{-1})	K_m (µM)	k_{cat}/K_m (µM^{-1} min^{-1})
wt	0.0063 ± 0.0006	8.4 ± 0.7	0.00075 ± 0.00009
R106K	0.0122 ± 0.0004	9.9 ± 1.2	0.0012 ± 0.00015
R174K	N/A	N/A	N/A
M60L	0.0017 ± 0.0002	10.0 ± 3.5	0.00017 ± 0.00006
M166A	0.00031 ± 0.00003	40 ± 19	0.0000077 ± 0.0000037
R106K/R174K	0.0188 ± 0.0005	10.1 ± 1.1	0.0018 ± 0.0001

The effect of the mutants on PRMT6 activity on H3 (1-31) peptide showed that mutation of R106 and R174 can lead to different regulation strategies on its substrate. R174K showed no activity for the substrate. Perhaps the ability to automethylate at this point serves to activate PRMT6 for catalysis. As

seen in Figures 3.17, the loss in ability to automethylate can be related the lack of activity on H3 (1-31). However, R174 is a conserved residue and as discussed previously could be fatal if mutated. Methylation at R106 could suppress activity for catalysis. Methylation of Arg can cause steric hindrance and disrupt H-bonding to affect substrate binding. PRMT1 is the predominant type I PRMT in vivo. It is likely that PRMT1 can methylate PRMT6 in order to repress the activity of PRMT6 in vivo. M60L and M166A showed very little activity proving their importance to catalytic activity which is consistent with prior studies of alteration in substrate specificity [33]. Further evidence that these locations are important is their conservation among different species PRMTs. Interestingly, R106K/R174K showed higher activity. This could be associated to the alteration in the overall structure of PRMT6 SAM binding domain. Further investigation of the effects that the R106K/R174K mutant has on the structure is required to make conclusions.   PRMT6-R174K will need additional testing on multiple substrates to conclude if the enzyme retains methyltransferase activity.

3.1.8   *Tag free PRMT1 behaves similarly to His-PRMT1 in PRMT6 methylation*

The experiment was performed to determine if the use of tf- and gst-PRMT1 were good options for the isolation of methylated PRMT6 using the nickel bead quenching method. PRMT1 with/without tags was used to methylate H4, H4-20, and PRMT6 to determine the effects of the tag on activity. The reactions were visualized using SDS-PAGE staining and phosphorimaging. Previous experiments only utilized His-PRMT1 for methylation reactions. To determine if the same level of methylation can be obtained three gel assays were performed.
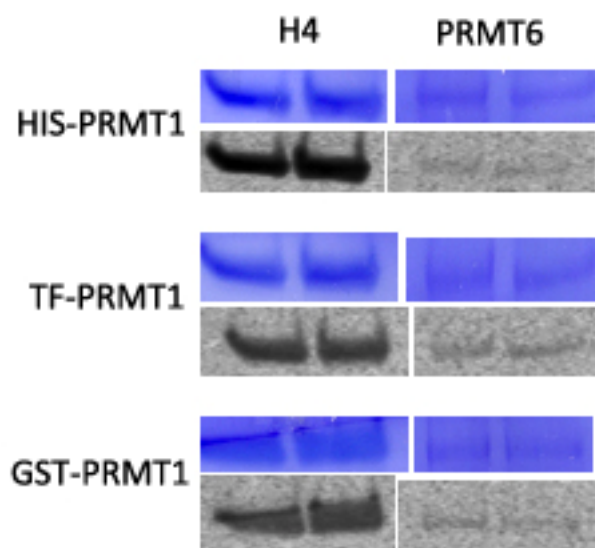
Figure 3.21- Coomassie stain and phosphorimage of PRMT1 mediated methylation of H4 and PRMT6. Unfortunately the H4-20 peptide was lost during staining/destaining. Only H4 protein methylation was maintained. PRMT6 methylation was very low due to the 15 min reaction time and the low loading amount.
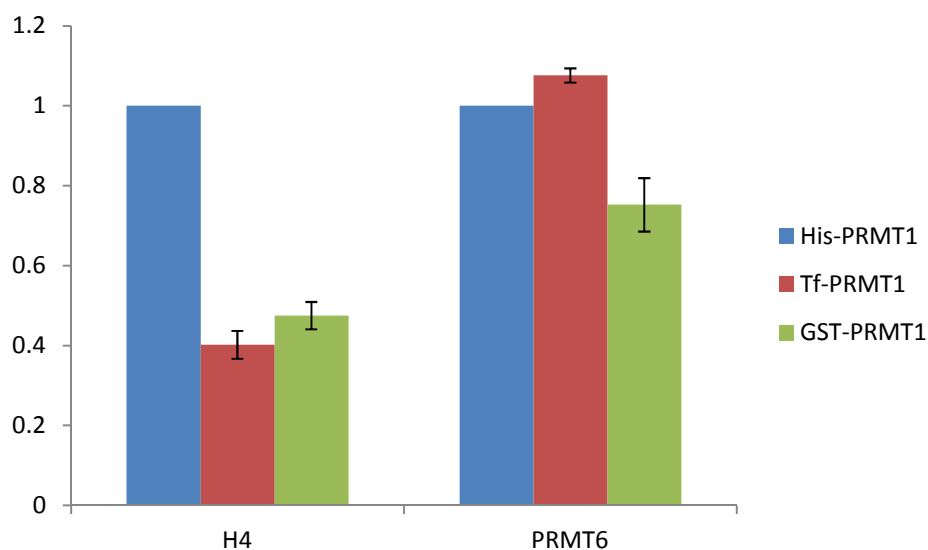
Figure 3.22- Normalized methylation levels to His-PRMT1

The gel was analyzed with QuantityOne software and the results were normalized to the His-

PRMT1 in order to relate to previous experiments (Figure 3.22). Both tf- and GST-PRMT1 show the abil-

ity to methylate the H4 protein, however, the methylation levels are ~50% that of the His-PRMT1. H4-20

methylation was not obtained for the GST-PRMT1 due to the loss of the peptide after destaining. Prior

experiments show that the methylation of the H4(1-20) by tf- was ~50% that of His-PRMT1. PRMT1 me-

diated methylation of PRMT6 show similar methylation levels. GST-PRMT1 showed ~80% from His-

PRMT1. Tf-PRMT1 showed slightly more methylation than His-PRMT1.

The concept of using GST-PRMT1 and tf-PRMT1 is to carry out a methylation reaction on His-

PRMT6 and use Ni-beads to quench and isolate the methylated PRMT6. The molecular weight of GST-

PRMT1 is 68 KDa. GST-PRMT1 used in the isolation of methylated PRMT6 would cause issues due to the

impurity band near 66 KDa shown in PRMT6 SDS-PAGE(Figure 3.10). Tf-PRMT1 would be ideal since it

can be clearly distinguished from PRMT6. From the data, tf-PRMT1 may be able to methylate PRMT6 in

an identical way to that of His-PRMT1. A product formation assay to validate this experiment is neces-

sary to carry on towards the isolation of methylated PRMT6.

3.2   DMI Inhibits Oligomerization of PRMT1

3.2.1   *Dimerization inhibitors successfully synthesized*

The DMI peptides were designed from the sequence of the dimerization arm of PRMT1. The

peptides were synthesized on a PS3 peptide synthesizer using the Fmoc strategy SPPS. The synthesized

peptides checked by MALDI after resin cleavage showed that the peptides were successfully synthesized

(Appendix). DMI 22 was very difficult to dissolve for HPLC purification. So DMI 26 was designed to de-

crease the hydrophobic properties. During purification the gradient used was 75-85% ddH$_2$O and

acetonitrille with 0.5%TFA. The peaks observed eluted in a merged fashion and fractions of the peaks

were taken to determine the retention time containing the most pure peptide as seen by MALDI. Final

products were lyophilized and store in -20°C. A stock was prepared for testing. See appendix for chroma-

tograms and mass spectrums.

Table 3.2- DMI peptide sequence and mass. Both peptides successfully synthesized according to initial MALDI after resin cleavage. After RP-HPLC purification, a pure DMI 26 was obtained. DMI 22 was obtained after RP-HPLC, however, the solution was not pure.

Name	Sequence	Expected Mass	Observed Mass
DMI 22	Ac-KIHWWENVYGFDMSCIKDVAIK-H	2725	2725.6
DMI 26	Ac-KDYKIHWWENVYGFDMSCIKDVAIKE-H	3260.74	3260

3.2.2   Oligomerization of PRMT1 was inhibited by DMI26

The purpose of the experiment was to determine the effective concentration of DMI26 for inhibition. PRMT1 was incubated with increasing concentrations of DMI overnight and then crosslinked with glutaraldehyde for Western blot visualization. Ratios of antibody use had to be optimized in order to visualize DMI effects. Oversaturation was observed with 1 hour antibody incubations. Ratios of primary antibody 1:2500 and secondary antibody of 1:6250 showed a slight loss in oligomers. Decreasing the antibody incubations to 45 min and increasing ratios of primary antibody 1:6000 and secondary antibody 1:15000 showed a distinct loss of oligomers over an increasing concentration of DMI.
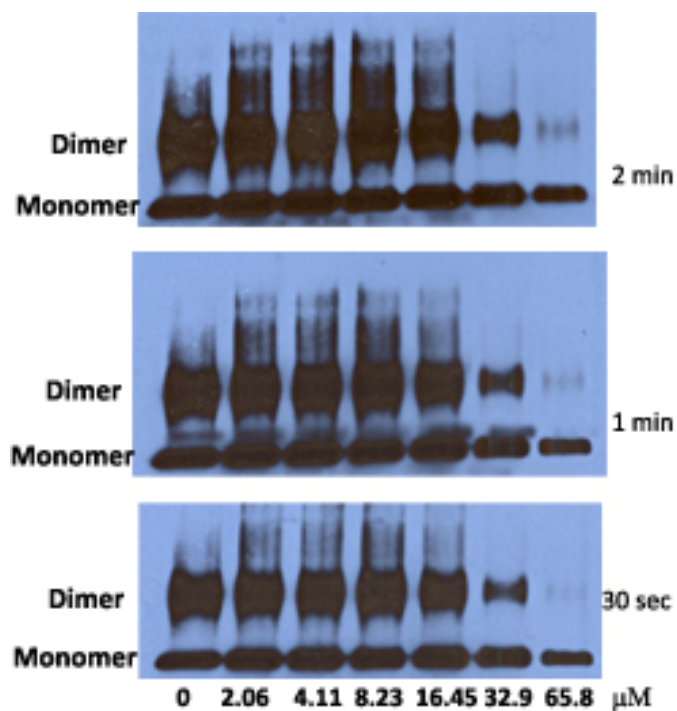
Figure 3.23- Western blot of PRMT1 with increasing DMI. Dimer and monomeric forms of PRMT1 were observed on the X-ray film. Exposure levels after 30 sec to the X-ray film show saturation. Initial loss of dimerization can be seen at 32.9 μM DMI. Near total loss is seen with 65.8 μM DMI.

Near complete loss of the dimer was observed at DMI 65.8 μM. Slight saturation of the film makes it difficult to determine the levels of the loss. The concentrations of the DMI were increased to allow us to visualize the gradual decrease in the oligomeric states of PRMT1. The antibody ratios were altered to reduce the saturation.
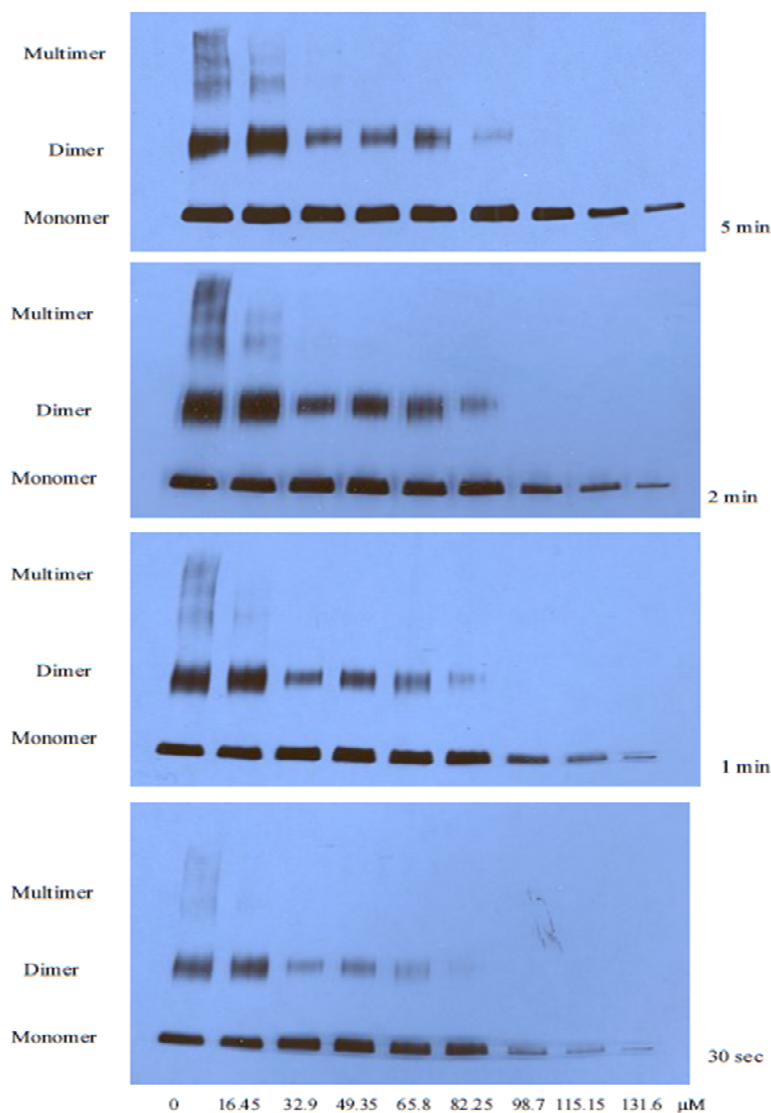
Figure 3.24- Western blot of PRMT1 with adjusted DMI range. The multimer, dimer, and monomer of PRMT1 can be seen with the increase of DMI concentration. Exposure times were from 30 sec to 5 min to detect oligomeric forms. Saturation levels were low enough for quantification.

The gradual loss of oligomers as DMI concentration increased can be clearly seen. At 16.45 μM remaining oligomeric states were 33%. Only 4% remained at 32.9 μM. No measurable amount of oligomerization could be obtained past 32.9 μM. For the dimer loss is first observed at 32.9 μM with 50% of the dimeric state can be observed till 65.8 μM. 11% of the dimer remained at 82.25 μM and total loss was observed at 98.7 μM with only 0.4%. Interestingly the monomer also showed a gradual de-

crease as the ability to dimerize is inhibited. The monomer remained relatively at 100% until 98.7

μM where 68% remained. 47% remained at 115.15 μM and 28% remained at 131.6 μM. Precipitant in a

tube of treated PRMT1 with 100 μM DMI after centrifugation supports that the monomer is not stable

and dimerization is needed for enzyme stability in vitro. Another possibility is that the DMI-26 causes

PRMT1 to crash out of solution. The monomeric for decreases after oligomeric PRMT1 cannot be de-

tected. A gradual increase or constant state of the monomer should have been observed. It is inconclu-

sive to state that the DMI-26 is really inhibiting oligomerization without further testing.

3.2.3   Dimer inhibition occurs within 2 hours with DMI

The optimal time conditions for PRMT1 and DMI interaction was determined by Western blot.

PRMT1 was incubated with DMI for a period of 12 hours and were cross linked and flash frozen with liq-

uid nitrogen till visualization using Western blotting (Figure 3.25). The chosen 100 μM of DMI was used

to completely inhibit dimerization. At 2 hours there is an observed decrease in the dimerization. Expo-

sure times of 30 sec show complete loss at 2 hours. 2 min exposure time shows a gradual decrease of

dimerization from 0-8 hours with a complete loss at 10 hours. For the purpose of the kinetic studies 3-4

hour incubation would be utilized in order to characterize the activity of PRMT1 with DMI assuming that

there is a loss in dimerization.
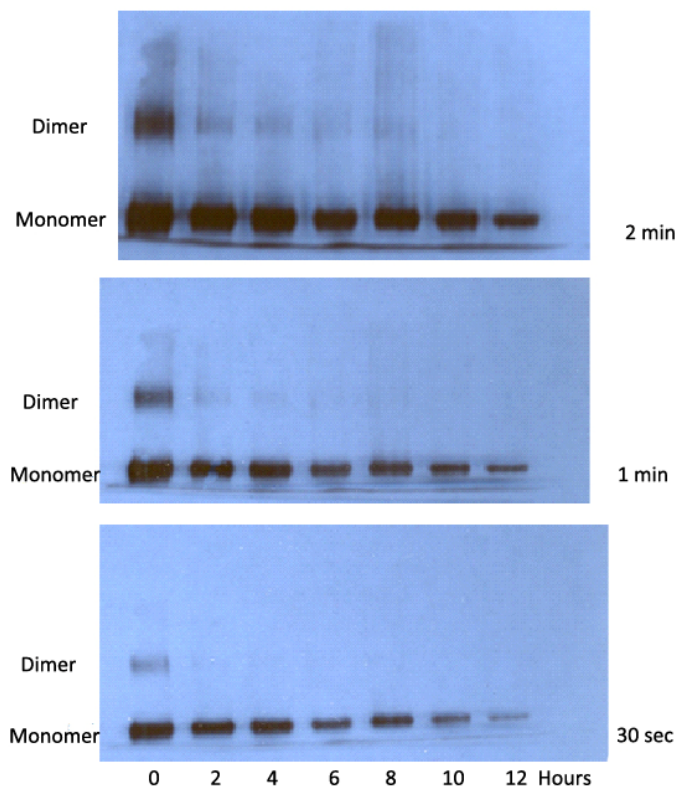
Figure 3.25- Western blot for optimal DMI/PRMT1 incubation times

The reactions were quenched over a period of 12 hours to emulate the conditions that were

used prior. As can be seen in the figure 50% of dimers remain 2 hours into the incubation. 4 hours into

incubation the dimer is not apparent after 30 sec exposure and 1 min exposure shows 15%. The remain-

ing dimer for 6-12 hours were >10%. The monomer decreased to 85%-95% at 2-4 hours. The monomer

showed >60% after 6 hours. This shows that the peptide is efficient at short times and can effectively

produce monomer forms of PRMT1. The 4 hour incubation time was used for kinetic assays to reduce

the loss of the monomer form. The monomer still shows a decrease over time further supporting its in-

stability in vitro when not oligomerized. However, we cannot rule out the possibility that DMI-26 is caus-

ing PRMT1 to crash out of solution.

3.2.4 *TFE is lethal to PRMT1 activity*

The DMI peptide was synthesized according the dimerization arm sequence of PRMT1 which consist of a α-helix-loop- α-helix motif. TFE has been shown to have the ability to stabilize/induce secondary structure of proteins and peptides.TFE was initially used to stabilize the secondary structure of the peptide to ensure that the peptide will interact with the dimerization interface. The solubility issues faced when dissolving the peptides also support the existence of secondary structure. Low percentages of TFE show drastic affects on PRMT1 activity on H4 protein (Figure 3.26).
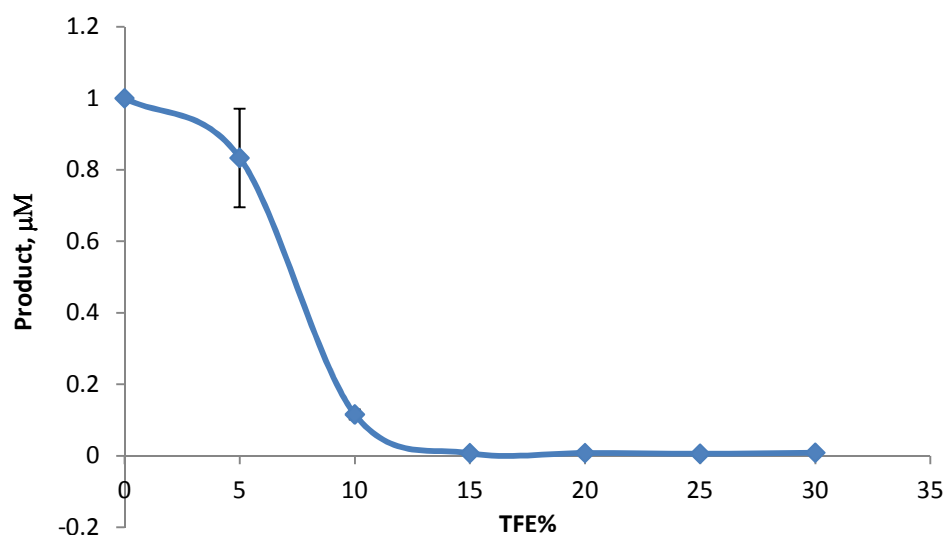
Figure 3.26- TFE dramatically decreases PRMT1 activity on H4. PRMT1was reacted with H4 protein with increasing TFE from 0-30% . The reaction occurred for 1 hour.

80% of activity was retained at 5% TFE. There is a complete loss in activity from 15%-30%. The option of using TFE to stabilize the DMI structure could compromise and interfere with activity and kinetic assays. The overall flexibility of the enzyme could be inhibited dirupt SAM binding. TFE was excluded from further experiments due to its lethality.

3.2.5 *DMI shows no effect on PRMT1 activity at initial dimerization disruption*

The experiment was performed to test the effects of PRMT1 activity at initial stages of inhibition of oligomerization by DMI. The assay was performed by incubating PRMT1 with DMI for 4 hours prior to

reaction initiation with H4 protein and ^{14}C-SAM and product formation was tracked from 0-120 min

(Figure 3.17). From the data obtained from the activity assay there was no significant difference in

PRMT1's ability to produce product over time. From the Western blot after 16.45 μM DMI oligomeric

states were decreased and total loss was observed at 32.9 μM DMI and a gradual decrease in dimer was

observed from 16.45 μM to 98.7 μM DMI.
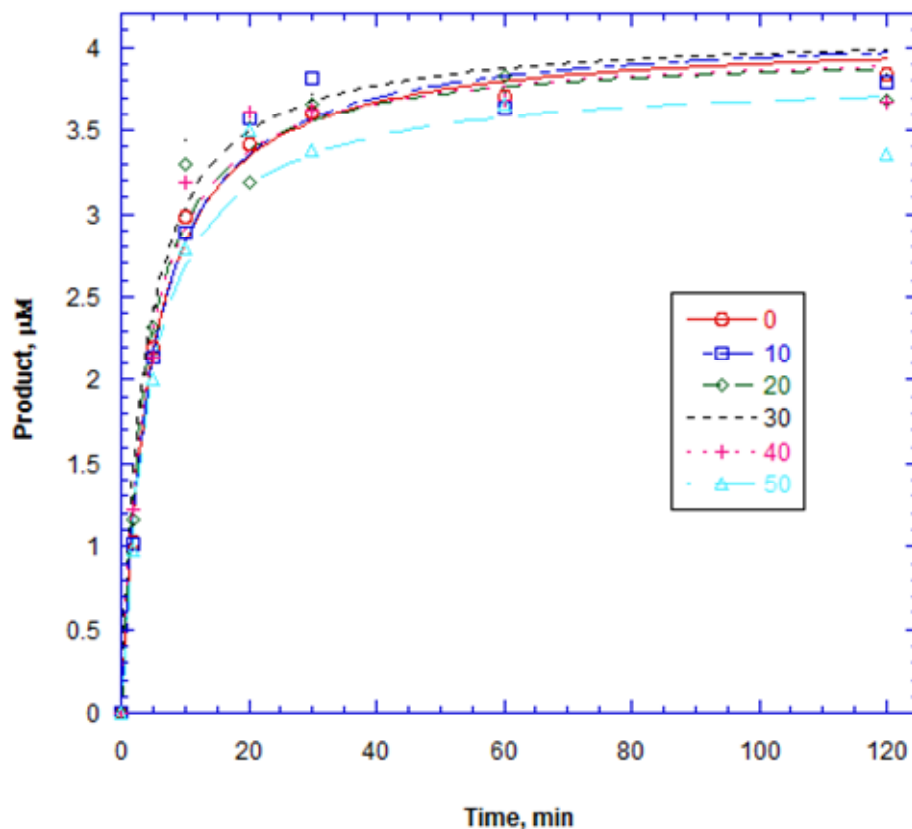
Figure 3.27- Product formation by PRMT1 with DMI. Product formation after initial inhibition by different concentrations of DMI26 (0, 10, 20, 30, 40, 50 uM) was tracked from 0-120 min. Time points were quenched on p81 filter paper at each time point. H4 protein was used as methyl acceptor and ^{14}C-SAM was the methyl donor.

From the data no significant shifts can be observed. DMI26 has no effect on the activity of

PRMT1 at low inhibition levels. The oligomerization of PRMT1 is first disrupted at low concentration of

DMI. From the data it seems that the inhibition of higher order oligomers does not affect the activity.

Half of the dimeric is retained from 40-50 μM DMI and the data remains relatively close to that of un-

treated PRMT1.

3.2.6   *The presence of DMI26 does not affect for PRMT1 activity on H4-20 methylation*

DMI26 was tested to see if the loss of PRMT1 oligomerization can have an effect on PRMT1's ac-

tivity. PRMT1 was incubated with H4 (1-20) and DMI26 from 0-127 μM prior to reaction initiation with

[14]C-SAM. The concentration range was chosen from Western blot conditions. The data obtained shows

no loss in activity from DMI concentrations ranging from 21.2-127.2 μM (Figures3.28–3.29). There is a

very slight increase that is not significant enough to say it increases activity.
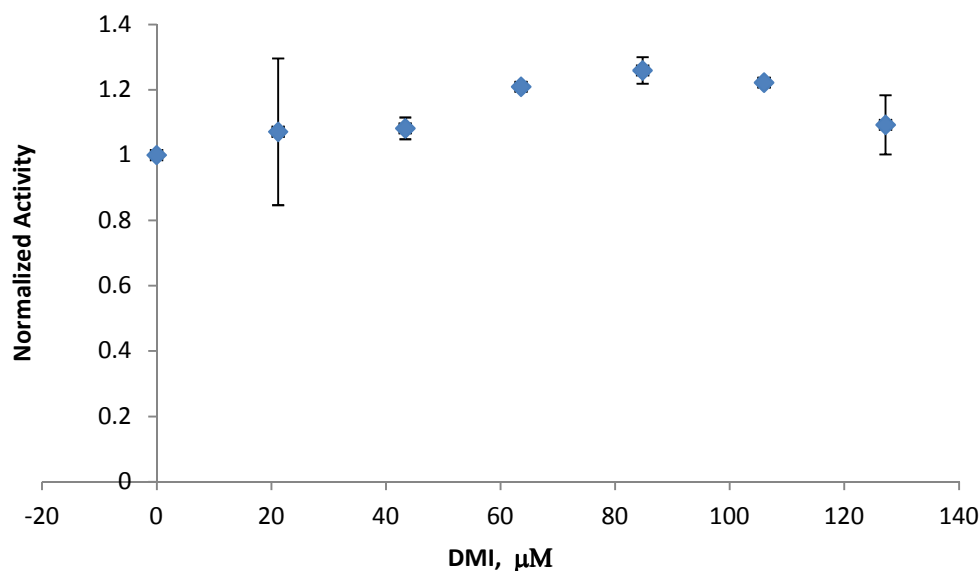
Figure 3.28- Effect on PRMT1 activity of DMI at varied concentrations.

The methylation reaction was carried out after an initial incubation to ensure DMI binding. The

measure activity was normalized to the PRMT1 activity with no DMI. There is error present in one point

due to insufficient washing for the p81 paper. Paper was stuck to the sides of the container.
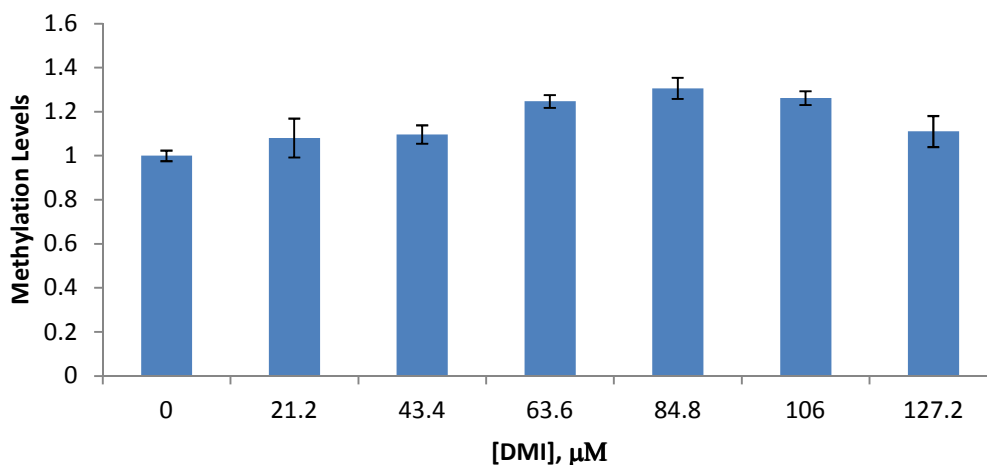
Figure 3.29- Effect on PRMT1 activity of DMI at varied concentrations. (bar graph)

The data was represented as a bar graph for better visualization that there is no significant effect on PRMT1 activity. This data suggests that the monomer retains activity that the oligomeric PRMT1 processes. This contradicts the belief that dimerization and oligomerization is necessary to methyltransferase activity .However, it must also be taken into account that binding substrates could affect the DMI binding. Further testing to investigate the effects of substrate binding to that of DMI/PRMT1 interaction must be performed.

3.2.7   *FPLC did not detect difference in oligomeric state in the presence of DMI26*
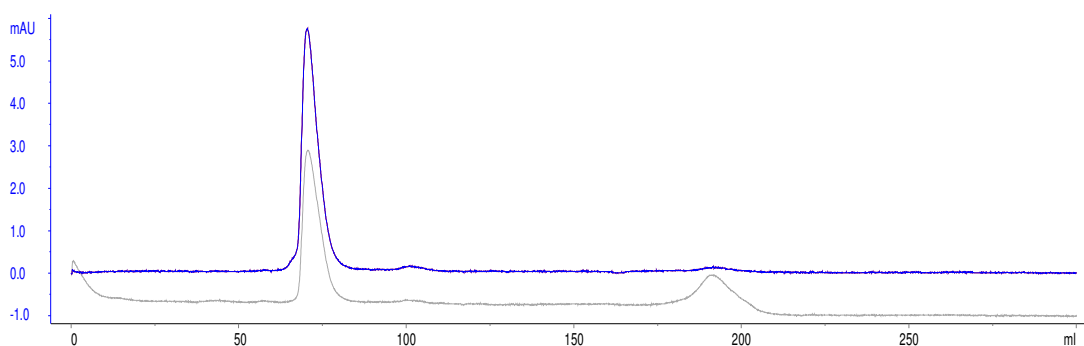
Figure 3.30- FPLC of PRMT1 with (grey colored) and without (blue colored) DMI.

FPLC was used to visualize the monomer and oligomer states of PRMT1 with/without DMI (Figure 3.30). PRMT1 was aged with DMSO or DMI for 4 hours prior to injection into the FPLC. The overlaid chromatograms matched each other really well. The blue chromatogram represents the control PRMT1. The grey line signifies the monomer PRMT1 with 100 μM DMI. The retention shown near 200 could be the DMI peptide. There is a slight rise in the control at this location as well showing it may be a combination of DMI and DMSO. FPLC was not able to show that DMI was isolating the PRMT1 monomer. Perhaps the binding of DMI will be broken upon injection since it gets diluted with FPLC running buffer. It also may be possible that the DMI is separated from PRMT1 when it enters the column. The pores can separate the peptide and PRMT1 will aggregate together. It is also possible that the signal is too low to differentiate the monomer from the dimer. A 100 μl sample of 20 μM PRMT1 was loaded to the loop and only 6 mAU was observed.

3.2.8   *Native gel analysis did not show different oligomeric resolving patterns in the presence of DMI26*

Native PAGE was used as another method to determine if the monomer and oligomer forms could identified when resolved natively. The effect of substrate binding on DMI/PRMT1 interaction was also tested. PRMT1 was incubated with/without DMI, SAM, and H4-20. The reactions were resolved on a 10% native SDS gel. The experiment was carried out similarly to the previous Western blots. No cross linking or heating was performed to ensure that the native structure remained intact. The samples chosen were a control PRMT1, DMI treated PRMT1 and a PRMT1 sample with DMI and SAM. The DMI and SAM samples were intended to analyze if SAM binding to the enzyme could disrupt the DMI binding. The gels were originally run at 4°C for 4.5 hours. After many trials this seemed unnecessary to continue at 4°C. Various voltage levels were tried to resolve the samples. The time for each run was tracked by watching the dye resolve to the bottom of the gel. The transfer to the membrane run and Western blot pro-

cedure was kept the same. Incubation times and ratios of antibodies were used at their optimized values

previously used.
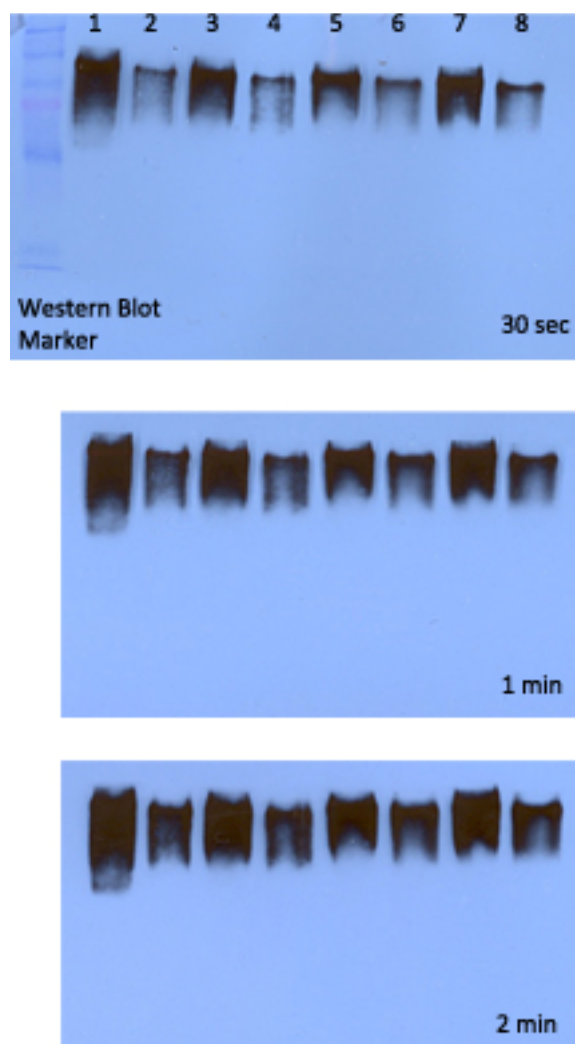
Figure 3.31- Native PAGE-Western blot of PRMT1 and DMI. There was not separation of the samples over a period of time. Lanes 1-2 contains only PRMT1. Lanes 3-4 contain PRMT1 and DMI. Lanes 5-6 contain PRMT1, DMI, and SAM. Lanes 7-8 contain PRMT1, DMI, and SAM added after initial incubation. Loading amounts for each sample were varied between 2 and 5 µl. There appears to be a slight smearing effect as the samples resolve. Lower sample loading amounts were used to reduce this smear effect. Western blot marker was loaded by lane 1 to gauge the migration of the samples.

A denatured Western blot marker resolved through the native gel but no distinct difference in

gel migration could be observed from the experiment. This result is consistent with the FPLC chromato-

gram which shows that PRMT1 with/without DMI will separate identically. Perhaps the DMI is disassoci-

ated from PRMT1 in the pore of the gel. A native gel was performed with the same parameter for check

the results obtained (Appendix: Figure SI-9). Unfortunately no distinct differences in gel migration could

be observed form the Western blot and further testing will be necessary to support DMI effectiveness in

isolating the monomeric form of PRMT1.

3.2.9 Steady state kinetic analysis of DMI treated PRMT1

The purpose of the experiment is to determine if the DMI treated PRMT1 will show the same ki-

netic parameters of the untreated PRMT1. PRMT1 was incubated with H4-20 and with/without DMI for

4 hours and the reaction was initiated with ^{3}H-SAM. A control was performed with DMSO instead of

DMI. Determining the kinetic parameters of the "monomeric PRMT1" on H4(1-20) methylation was real-

ly difficult. The experiment had to be adjusted for DMI/PRMT1 binding. ^{3}H-SAM was used to initiate the

reaction to ensure no degradation of SAM would occur during the 4 hour incubation. Error was extreme-

ly high for the initial experiments possibly due to the fluctuations of PRMT1 during the long incubation

times. The PRMT1 samples with DMI would be relatively unstable as shown in the figure 3.25. The factor

that the instability would have on the kinetics would be difficult to isolate from the effects of just the

monomeric state. The 4 hour incubation was used from the Western blot data which ~85% of monomer

is retained for 1 μM PRMT1. The assay used 0.4 μM PRMT1 which may have precipitate out more.
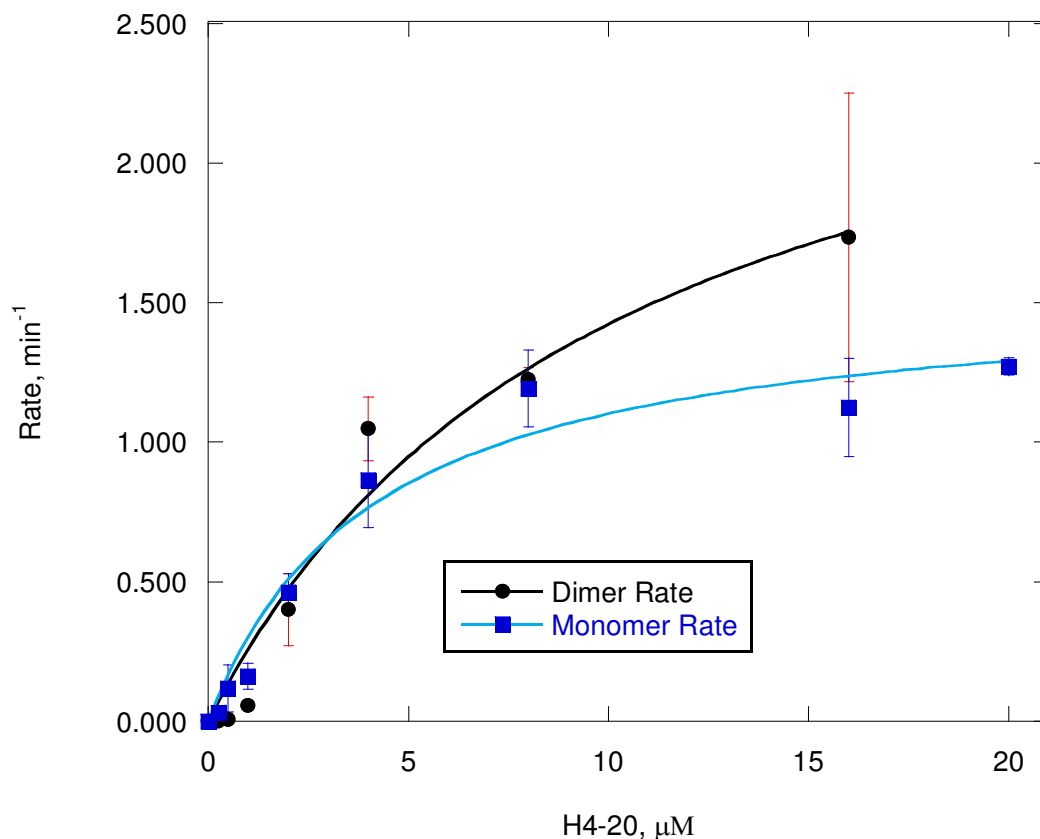
Figure 3.32- Steady state analysis of DMI treated/untreated PRMT1. PRMT1 was incubated with/without DMI for 4 hours prior to reaction initiation with ^{3}H-SAM. The control PRMT1 was incubated with the equivalence of DMSO. H4-20 range was from 0-20 μM for the 3 trials of the DMI treated PRMT1. H4-20 range was from 0-16 μM for the 2 trials with untreated PRMT1. The initial stages of the reaction did not exhibit steady state for the control. Saturation was not obtained for the 15 min reaction time.

The data was analyzed using KaleidaGraph to fit the data. The estimated parameters given were k_{cat}= 1.557 min^{-1} ± 0.1479, K_m= 4.1326 μM ± 1.1651, and k_{cat}/K_m=0.37676 μM^{-1}min^{-1}. The parameters given cannot be used for the actual values, but can be used as a general reference for comparison. The estimated k_{cat}/k_m using initial rates is 0.169 μM^{-1}min^{-1}. The control PRMT1 contained the same final

amount of DMSO as the DMI treated PRMT1. The incubation was carried out with the same conditions

to rule out the effect of loss of activity for long incubation periods. From the graph, PRMT1 still does not

reach saturation and initial stages do not show steady state. KaleidaGraph forced fit the data to obtain a

rough comparison to that of the "monomer". The estimated kinetic parameters obtained were kcat=

2.0346 min^{-1} ± 0.44.03, Km= 6.1771 μM ± 3.0144, and kcat/Km= 0.3294 μM^{-1}min^{-1}. Initial rates could not

be obtained since the data shows an exponential increase. The estimated data from kaleidagraph con-

tains a significant amount of error associated to the estimated parameters. From that data, there really

is not a significant difference in the activity of the uninhibited/inhibited PRMT1. This data supports that

the "monomer" could quite possibly retain function without the need to oligomerize. Refinement of the

assay would be needed to determine the exact kinetic parameters.

In previous studies using mutagenesis of the dimerization interface yielded decreased

methyltransferase activity showing that dimerization is essential for methyltransferase activity [34]. We

showed in this study that inhibition of homodimerization does not decrease methyltransferase activity.

The overall kinetic data supports that the oligomeric and monomeric rPRMT1 states will act with similar

activity. In both cases the catalytic efficiencies were nearly identical. The initial stages were taken for the

DMI treated PRMT1 to get k_{cat}/K_m. Due to difficulty in comparing the two results, we can speculate that

perhaps the monomeric form of PRMT1 can retain methyltransferase activity similar to that of the oli-

gomer. Inhibition of the homodimerization did not have a distinct effect on activity on H4(1-20) for

rPRMT1 suggesting that homodimerization is not essential for function. With the long incubations and

instability of the DMI treated PRMT1 it is difficult to optimize the assay for consistent results. Over the

long incubation time with DMI, PRMT1 will be unstable and precipitate out of solution. Initial stages for

the control failed to yield a consistent steady state. Likewise, saturation was not obtained. The error

seen in Figure 3.32 could be associated to the aging of the enzyme. We cannot omit the possibility that

substrate binding of either SAM or H4(1-20) can disrupt the DMI binding to promote dimerization. Fur-

ther tests to uncover the binding affinity of DMI to PRMT1and possible binding changes upon introduc-

tion of substrates are needed.

4   CONCLUSIONS

With the diverse functionality of PRMTs in biological systems it is important to understand how these enzymes function and intercommunicate. PRMT1 and PRMT6 are both type 1 PRMTs that share the ability to methylate histones and other protein substrates [35]. It has been questioned that this overlap in substrate specificity could allow for intercommunication of PRMTs via product formation. The only other interaction that has been shown for PRMTs is the formation of heterodimers between PRMT1 and PRMT2 [36]. For the first time, we report that PRMT1 interacts with and methylates PRMT6. We show that R106 is an important PRMT1 mediated methylation site of PRMT6 and R174 may be an important automethylation site of PRMT6. It should be noted that R174 is a conserved residue between PRMT1, PRMT2 and PRMT6. Further investigation of the residues importance to PRMT6 will be needed in order to make conclusions that the mutation is not fatal to the enzyme. The double mutation of PRMT6 at R174/R106 shows returns to normal PRMT1 mediated methylation and doubles the levels of automethylation. This may be because the mutation alters in the overall structure, thus affecting protein-protein interactions. R174 resides near the SAM binding pocket, so automethylation could easily occur at this site in the absence of other substrates. R106 is on the surface of the dimerization interface which can be easily accessed by PRMT1. Perhaps methylation of PRMT6 by PRMT1 can alter substrate binding of the enzyme. However, methylation of PRMT6 by PRMT1 did not show a very distinct effect on PRMT6 activity for H3 methylation (Appendix: Figure SI- 5-6). It is unknown if PRMT6 can be methylated by PRMT1 in the cell. The Coimmunoprecipitation experiment showed that they do interact but the level of interaction needs more study. Further studies will be necessary to examine the effects of PRMT6 automethylation and PRMT1 mediated methylation on PRMT6 methyltransferase activity on substrates. PRMT-4,-6 and -8 have the ability to automethylate, but the effects of automethylation is not understood. The fact that PRMT1 can methylate PRMT6 is the first report of PRMT methylation by another

PRMT. The consequence of this methylation may give insight to how PRMT6 activity can be modified with methylation by PRMT1.

All PRMTs process a conserved dimerization arm supporting that PRMTs can oligomerize [29, 31]. The DMI peptide was shown to be very effective for inhibiting PRMT1 homo-oligomerization according to the cross linking experiment. However, there were issues in further supporting this finding. The Western blot data obtained from both the time dependent and gradient incubation with DMI-26 and PRMT1 showed a gradual decrease in the monomeric PRMT1 making the data inconclusive of successful inhibition of oligomers. It is possible that the DMI causes PRMT1 to crash out of solution. It may also be possible that the monomer of PRMT1 is unstable causing it to degrade. Gel filtration and native PAGE were unsuccessful to detect the effect of DMI on PRMT1 oligomerization. From the preliminary data, the DMI treated PRMT1 seems to function identically to that of the untreated PRMT1. A previous study based on mutagenesis [29, 34] suggests that homooligomerization is essential to PRMT function which may not be the case if the DMI is isolating the monomer form of PRMT1. Though the dimerization arm is conserved in the PRMT family, perhaps oligomerization is only important for protein stability. DMI needs further testing to verify the results from the preliminary data obtained. Further studies are needed to determine if DMI is binding with PRMT1 on the SAM-binding domain. The inhibition of PRMT1 oligomerization would have to be repeated to obtain a constant or increasing monomer band on the Western blot. The oligomers show a decrease but the monomers do not show an increase. It is necessary to explain why there is a loss in all forms of PRMT1.

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APPENDIX

(A)

R82: Di-methylation: NWAALR(+28)GK
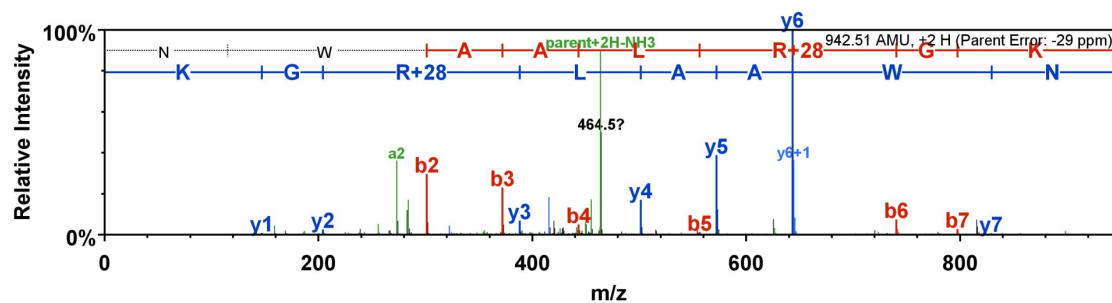
R174: Di-methylation: VHVLPGPVETVELPEQVDAIVSEWMGYGLLHESMLSSVLHAR(+28)
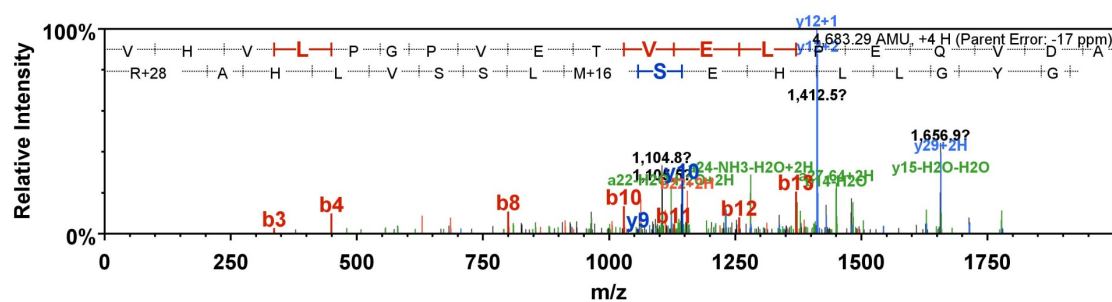
R29: Mono-methylation: KLESGGGGGEGGEGTEEEDGAER(+14)
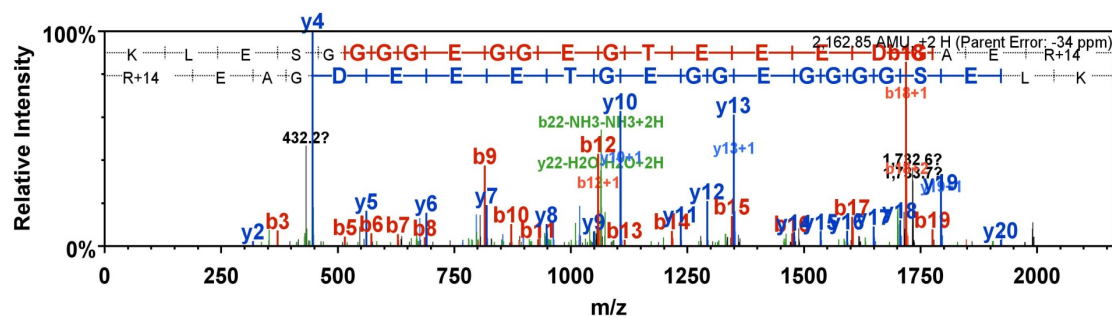
R106: Mono-Methylation: TVLDVGAGTGILSIFCAQAGAR (+14)
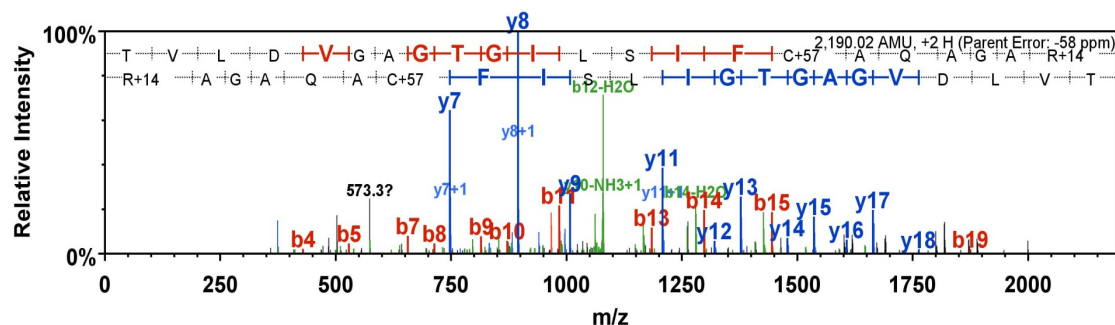
R228: Mono-methylation: QHYGVDMSCLEGFATR (+14)
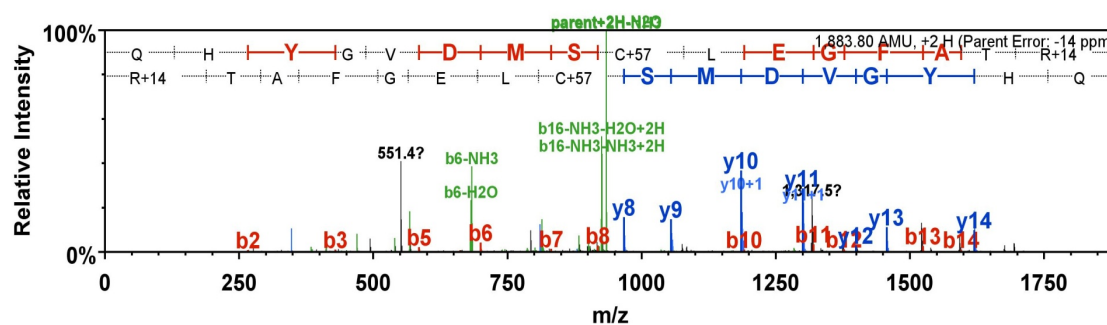
(B)

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MSQPKK RKLE   SGGGGEGGEG  TEEEDGAER E    AALERPRRTK   RERDQLYYE C
YSDVSVHEEM    IADRVRTDAY   RLGILRNWAA    LRGKTVLDVG   AGTGILSIF C
AQAGA R RVYA  VEASAIWQQA   REVVRFNGLE   DRVHVLPGPV   ETVELPEQVD
AIVSEWMGYG    LLHESMLSSV   LHAR TKWLKE   GGLLLPASAE   LFIAPISDQ M
LEWRLGFWSQ    VKQHYGVDM S  CLEGFATR C L  MGHSEIVVQG   LSGEDVLARP
QRFAQLELSR    AGLEQELEAG   VGGR FRCSCY   GSAPMHGFAI   WFQVTFPGGE
SEK PLVLSTS   PFHPATHWKQ   ALLYLNEPVQ   VEQDTDVSGE   ITLLPSR DNP
RRLRVLLR YK   VGDQEEKTKD   FAMED
```

Figure SI-1. LC-MS/MS of Methylated PRMT6

PRMT1, PRMT6, and SAM were incubated at 30°C for 3 hours. Reactions were quenched with SDS loading dye and resolved on SDS-PAGE. The PRMT6 band was cut out and vacuum dried. The gel band was sent to UAB Proteomics & Mass Spectrometry Facility for analysis. The gel was digested with trypsin and analyzed with MS/MS. The resulting modified peptides were reported with the mass spectrum and highlighted overlay on the sequence.

(A) PRMT6-R29K

PRMT6(R29K)f: **gaagatggcg cggagaagga ggcggccctg gag**

PRMT6(R29K)r: **ctccagggccgcctccttctccgcgccatcttc**

(B) PRMT6-R82K

PRMT6(R82K)f: **ga actgggcagc actgaaaggc aagacggtac tgg**

PRMT6(R82K)r: **ccagtaccgtcttgcctttcagtgctgcccagttc**

(C) PRMT6-R106K

PRMT6(R106K)f: **cccaggcc ggggccaagc gcgtgtacgc gg**

PRMT6(R106K)r: **ccgcgtacacgcgcttggccccggcctggg**

(D) PRMT6-R228K

PRMT6(R228K)f: **ggag ggcttcgcca cgaagtgtct catgggccac tc**

PRMT6(R228K)r: **gagtggcccatgagacacttcgtggcgaagccctcc**

(E) PRMT6-R174K

PRMT6(R174K)f: **gctccgt cctccacgcg aaaaccaagt ggctgaagg**

PRMT6(R174K)r: **ccttcagccacttggttttcgcgtggaggacggagc**

(F) PRMT6-M60L

PRMT6(M60L)f: **cggtcc acgaggagttgatcgcggac cgc**

PRMT6(M60L)r: **gcggtccgcgatcaactcctcgtggaccg**

(G) PRMT6-M166A

PRMT6(M166A)f: **ctcctgcac gagtccgcgc tgagctccgt cctc**

PRMT6(M166A)r: **gaggacggagctcagcgcggactcgtgcaggag**

Figure SI-2. Primer Design for PRMT6 Mutants
Primers designed to have Tm of 78^{o}C. Forward and reverse primers designed for site directed mutagenesis using the PRMT6 plasmid as a DNA template.

(A) PRMT6 mutant alignment (Combined T7/T7 term)

R29K T7-T7 term

```
Query   1    ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcggggggcggcggcgaaggaggggaggga   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   159  ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGCGGCGGCGAAGGAGGGGAGGGA   218

Query   61   actgaagaggaagatggcgcgcggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   219  ACTGAAGAGGAAGATGGCGCGCGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG   278
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Query   121   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   279   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   338

Query   181   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   339   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   398

Query   241   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   399   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   458

Query   301   GCCCAGGCCGGGGCCAAGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   459   GCCCAGGCCGGGGCCAAGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   518

Query   361   CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG   420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   519   CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG   578

Query   421   GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA   480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   579   GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA   638

Query   481   CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG   540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   639   CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG   698

Query   541   GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG   600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   699   GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG   758

Query   601   CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC   660
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Sbjct   759   CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC   818

Query   661   TGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTGCAGGGA   720
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Sbjct   820   TGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTGCAGGGA   879

Query   829   TGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTC   888
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Sbjct   383   TGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTC   324

Query   889   CCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACT   948
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Sbjct   323   CCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACT   264

Query   949   CACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGAC   1008
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Sbjct   263   CACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGAC   204

Query   1009  GTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTG   1068
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   203   GTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTG   144

Query   1069  CTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA   1128
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Sbjct   143   CTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA   84
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M60L T7/T7 term

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Query   1     ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcggggggcggcggcgaaggagggggaggga   60
              ||||||||||||||||||||||||||||||||
Sbjct   160   ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGCGGCGGCGAAGGAGGGGAGGGA   219

Query   61    actgaagaggaagatggcgcggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG   120
```

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                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220    ACTGAAGAGGAAGATGGCGCGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG    279

Query  121    CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGTTG    180
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280    CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGTTG    339

Query  181    ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA    240
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340    ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA    399

Query  241    CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT    300
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400    CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT    459

Query  301    GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC    360
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460    GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC    519

Query  361    CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG    420
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  520    CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG    579

Query  421    GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA    480
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  580    GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA    639

Query  481    CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG    540
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  640    CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG    699

Query  541    GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG    600
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  700    GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG    759

Query  601    CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC    660
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  760    CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC    819

Query  661    TGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTGCAGGGA    720
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  820    TGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTGCAGGGA    879

Query  873    GTTCCAGGTGACCTTCCCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCC    932
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340    GTTCCAGGTGACCTTCCCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCC    281

Query  933    TTTTCACCCGGCCACTCACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGT    992
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280    TTTTCACCCGGCCACTCACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGT    221

Query  993    GGAGCAAGACACGGACGTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCG    1052
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220    GGAGCAAGACACGGACGTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCG    161

Query  1053   TCGCCTGCGCGTGCTGCTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTT    1112
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160    TCGCCTGCGCGTGCTGCTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTT    101

Query  1113   TGCCATGGAGGACTGA    1128
                 ||||||||||||||||
Sbjct  100    TGCCATGGAGGACTGA    85
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R82K T7/T7 term

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Query  1      ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcgggggggcggcggcgaaggaggggaggga    60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158    ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGGCGGCGGCGAAGGAGGGGAGGGA    217
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Query  61   actgaagaggaagatggcgcgggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  218  ACTGAAGAGGAAGATGGCGCGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG  277

Query  121  CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278  CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG  337

Query  181  ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338  ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA  397

Query  241  CTGAAAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  CTGAAAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT  457

Query  301  GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458  GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC  517

Query  361  CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG  577

Query  421  GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  578  GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA  637

Query  481  CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638  CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG  697

Query  541  GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698  GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG  757

Query  601  CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758  CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC  817

Query  635   AGCAGCACTATGGTGTGGACATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGG  694
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575   AGCAGCACTATGGTGTGGACATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGG  516

Query  695   GCCACTCGGAGATCGTTGTGCAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGC  754
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   GCCACTCGGAGATCGTTGTGCAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGC  456

Query  755   GCTTTGCTCAGCTAGAGCTCTCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGG  814
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GCTTTGCTCAGCTAGAGCTCTCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGG  396

Query  815   GCGGGCGCTTCCGCTGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGT  874
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395   GCGGGCGCTTCCGCTGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGT  336

Query  875   TCCAGGTGACCTTCCCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTT  934
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335   TCCAGGTGACCTTCCCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTT  276

Query  935   TTCACCCGGCCACTCACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGG  994
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  275    TTCACCCGGCCACTCACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGG  216

Query  995    AGCAAGACACGGACGTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTC  1054
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215    AGCAAGACACGGACGTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTC  156

Query  1055   GCCTGCGCGTGCTGCTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTG  1114
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  155    GCCTGCGCGTGCTGCTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTG  96

Query  1115   CCATGGAGGACTGA  1128
              ||||||||||||||
Sbjct  95     CCATGGAGGACTGA  82
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R106K T7/T7 term

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Query  1      ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcggggggcggcggcgaaggaggggaggga  60
              ||||||||||||||||||||||||||||||||
Sbjct  159    ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGCGGCGGCGAAGGAGGGGAGGGA  218

Query  61     actgaagaggaagatggcgcgggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG  120
                                                  ||||||||||||||||||||||||
Sbjct  219    ACTGAAGAGGAAGATGGCGCGGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG  278

Query  121    CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  279    CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG  338

Query  181    ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  339    ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA  398

Query  241    CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  399    CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT  458

Query  301    GCCCAGGCCGGGGCCAAGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  459    GCCCAGGCCGGGGCCAAGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC  518

Query  361    CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  519    CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG  578

Query  421    GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  579    GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA  638

Query  481    CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  639    CTCCTGCACGAGTCCATGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG  698

Query  541    GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  699    GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG  758

Query  601    CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  759    CTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGACATGAGC  818

Query  649    GTGGACATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATC  708
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  563    GTGGACATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATC  504

Query  709    GTTGTGCAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGCGCTTTGCTCAGCTA  768
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct   503   GTTGTGCAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGCGCTTTGCTCAGCTA   444

Query   769   GAGCTCTCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGGGCGGGCGCTTCCGC   828
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   443   GAGCTCTCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGGGCGGGCGCTTCCGC   384

Query   829   TGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTC   888
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   383   TGCAGCTGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTC   324

Query   889   CCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACT   948
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   323   CCTGGAGGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACT   264

Query   949   CACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGAC   1008
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   263   CACTGGAAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGAC   204

Query   1009  GTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTG   1068
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   203   GTTTCAGGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTG   144

Query   1069  CTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA   1128
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   143   CTGCGCTACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA   84
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M166A T7/T7 term

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Query   1     ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcgggggggcggcggcgaaggaggggaggga   60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   165   ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGCGGCGGCGAAGGAGGGGAGGGA   224

Query   61    actgaagaggaagatggcgcgcggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG   120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   225   ACTGAAGAGGAAGATGGCGCGCGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG   284

Query   121   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   285   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   344

Query   181   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   345   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   404

Query   241   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   405   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   464

Query   301   GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   465   GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   524

Query   361   CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG   420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   525   CGGGAGGTGGTGCGGTTCAACGGGCTGGAGGACCGGGTGCACGTCCTGCCGGGACCAGTG   584

Query   421   GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA   480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   585   GAGACTGTAGAGTTGCCGGAACAGGTGGATGCCATCGTGAGCGAGTGGATGGGCTACGGA   644

Query   481   CTCCTGCACGAGTCCGCGCTGAGCTCCGTCCTCCACGCGCGCGAACCAAGTGGCTGAAGGAG   540
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               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  645   CTCCTGCACGAGTCCGCGCTGAGCTCCGTCCTCCACGCGCGAACCAAGTGGCTGAAGGAG   704

Query  541   GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG   600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  705   GGCGGTCTTCTCCTGCCGGCCTCCGCCGAGCTCTTCATAGCCCCCATCAGCGACCAGATG   764

Query  595    CAGATGCTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGAC   654
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  615    CAGATGCTGGAATGGCGCCTGGGCTTCTGGAGCCAGGTGAAGCAGCACTATGGTGTGGAC   556

Query  655   ATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTG   714
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555   ATGAGCTGCCTGGAGGGCTTCGCCACGCGCTGTCTCATGGGCCACTCGGAGATCGTTGTG   496

Query  715   CAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGCGCTTTGCTCAGCTAGAGCTC   774
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495   CAGGGATTGTCCGGCGAGGACGTGCTGGCCCGGCCGCAGCGCTTTGCTCAGCTAGAGCTC   436

Query  775   TCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGGGCGGGCGCTTCCGCTGCAGC   834
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435   TCCCGCGCCGGCTTGGAGCAGGAGCTGGAGGCCGGAGTGGGCGGGCGCTTCCGCTGCAGC   376

Query  835   TGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTCCCTGGA   894
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375   TGCTATGGCTCGGCGCCCATGCATGGCTTTGCCATCTGGTTCCAGGTGACCTTCCCTGGA   316

Query  895   GGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACTCACTGG   954
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315   GGGGAGTCGGAGAAACCCCTGGTGCTGTCCACCTCGCCTTTTCACCCGGCCACTCACTGG   256

Query  955   AAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGACGTTTCA   1014
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255   AAACAGGCGCTCCTCTACCTGAACGAGCCGGTGCAAGTGGAGCAAGACACGGACGTTTCA   196

Query  1015  GGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTGCTGCGC   1074
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195   GGAGAGATCACGCTGCTGCCCTCCCGGGACAACCCCCGTCGCCTGCGCGTGCTGCTGCGC   136

Query  1075  TACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA    1128
               |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135   TACAAAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA    82
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R174K T7/T7 term

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Query  1     ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTcgggggggcggcggcgaaggagggaggga   60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165   ATGTCGCAGCCCAAGAAAAGAAAGCTTGAGTCGGGGGGCGGCGGCGAAGGAGGGGAGGGA   224

Query  61    actgaagaggaagatggcgcgggagcgggaggcggcCCTGGAGCGACCCCGGAGGACTAAG   120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225   ACTGAAGAGGAAGATGGCGCGGGAGCGGGAGGCGGCCCTGGAGCGACCCCGGAGGACTAAG   284

Query  121   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285   CGGGAACGGGACCAGCTGTACTACGAGTGCTACTCGGACGTTTCGGTCCACGAGGAGATG   344

Query  181   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345   ATCGCGGACCGCGTCCGCACCGATGCCTACCGCCTGGGTATCCTTCGGAACTGGGCAGCA   404
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Query  241   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405   CTGCGAGGCAAGACGGTACTGGACGTGGGCGCGGGCACCGGCATTCTGAGCATCTTCTGT   464

Query  301   GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465   GCCCAGGCCGGGGCCCGGCGCGTGTACGCGGTAGAGGCCAGCGCCATCTGGCAACAGGCC   524

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Query  508   GTCCTCCACGCGAAAACCAAGTGGCTGAAGGAGGGCGGTCTTCTCCTGCCGGCCTCCGCC   567
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R228K T7/T7 term

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Sbjct  132   AAGTGGGAGACCAGGAGGAGAAGACCAAAGACTTTGCCATGGAGGACTGA  83
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Figure SI-2. Blast Sequence Alignment of DNA Sequenced PRMT6 Mutants
DNA sequencing was performed by Genescript and GSU biology department. Plasmids obtained after site directed mutagenesis were expressed in XL1-Blue and DNA was extracted using Promega DNA purification kit. The DNA was sequenced with T7 promoter and T7 terminator. The resulting sequenced data was combined to show 100% sequence alignment with the intact mutation.

(A) PRMT1 crystal structure overlay with PRMT6
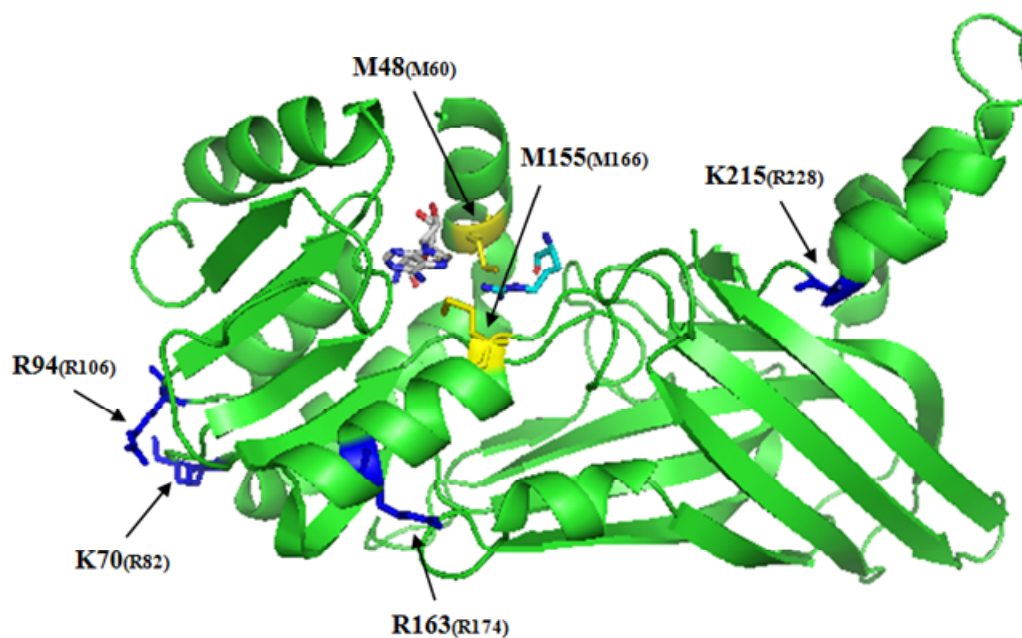
(B) Unpublished PRMT6 crystal structure (4HC4) with marked mutants
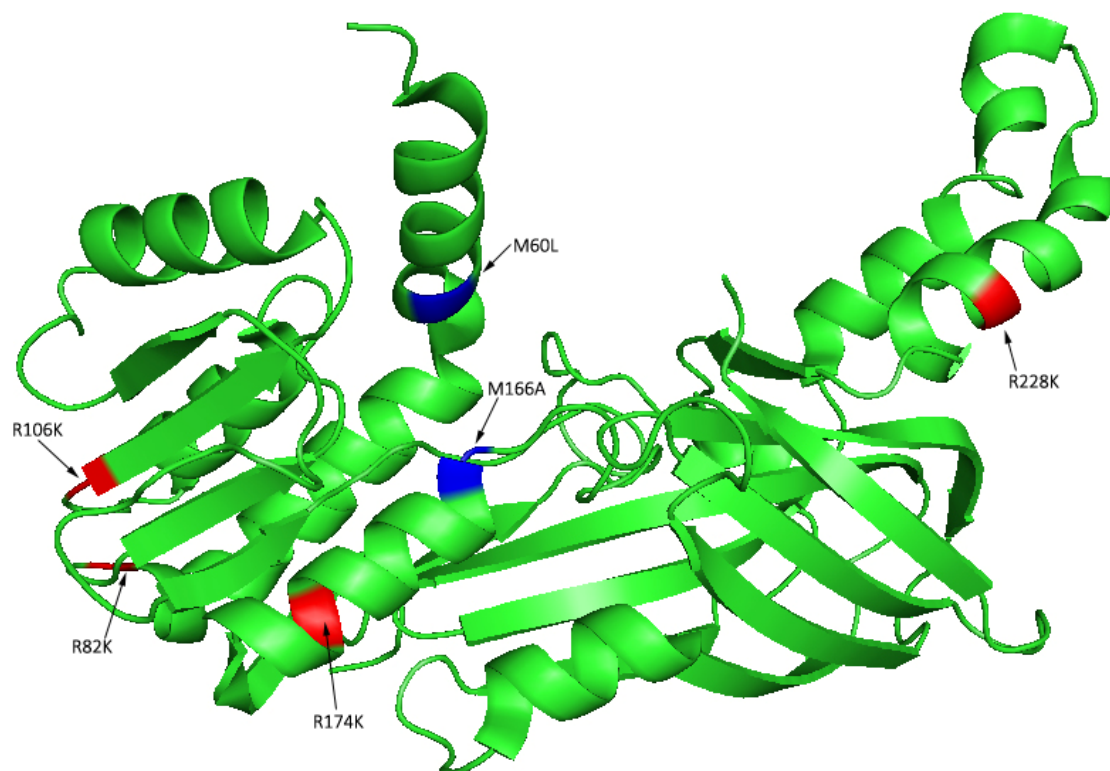
Figure SI-3. Crystal Structure examples of PRMT6 (pdb: 4HC4)
(A) The crystal structure of PRMT1 was used as a template for PRMT6 to show the general location of the mutations. (B)The mutations were color coded. Red represents an Arg to Lys mutation. The methionine mutants are shown in blue. The crystal structure is missing 40 amino acids in the N-terminal so R29 is not represented.

**(A) SDS PAGE of PRMT6 with H3 Protein then PRMT1**
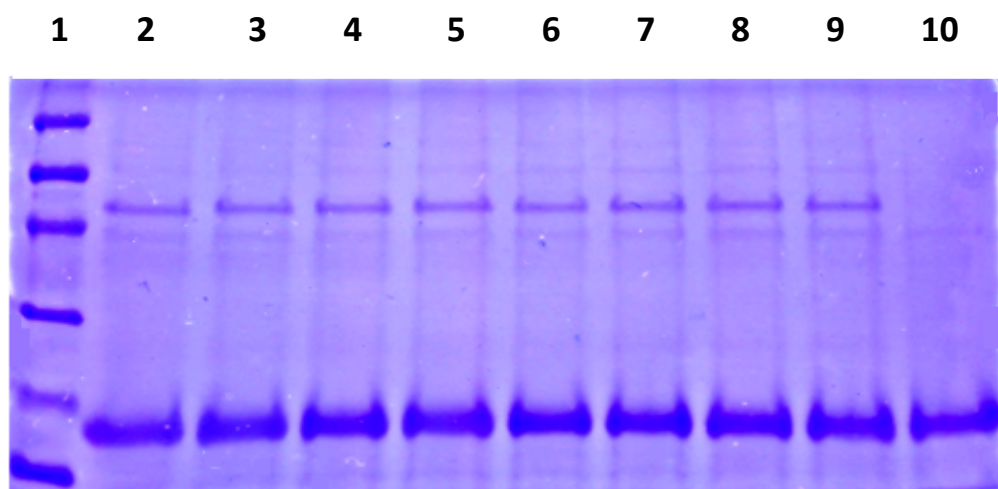
(B) Phosphorimage of PRMT6 with H3 Protein increasing PRMT1
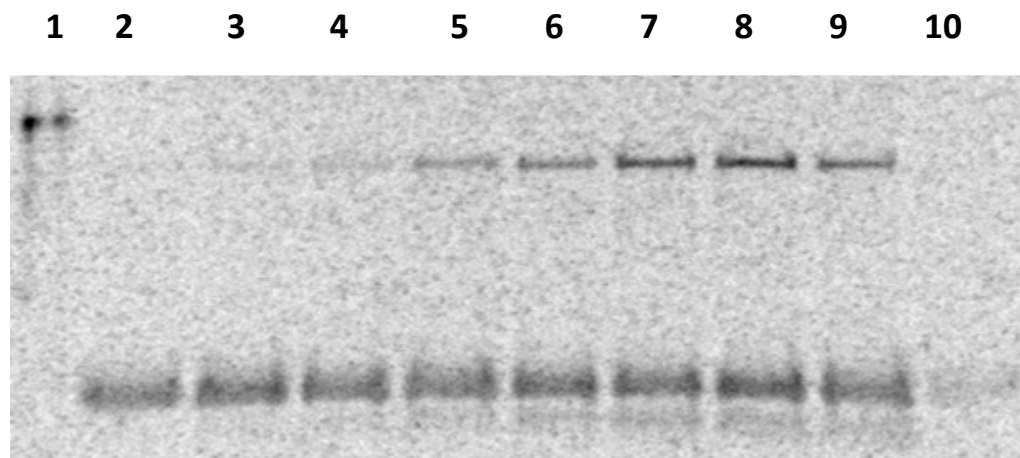
FigureS I-5. SDS and phosphor imaging of PRMT6 methylation by PRMT1 with H3 protein

(A) 4 μM PRMT6 was incubated with 0.5 μM PRMT1 and 25 μM ^{14}C-SAM for 0-4 hours. Then 5 μM

H3 protein and 50 μM and ^{14}C-SAM were placed into each reaction. The final concentrations shifted for

PRMT6 and PRMT1 to 0.8 μM and 0.1 μM respectively. The reaction was incubated for another 15 min

before it was quenched by SDS loading buffer. 15% SDS-PAGE was used to resolve the reaction mix-

tures and the gel was stored 48 hours in the phosphorcassete. (1) makers; (2) ~ (9): PRMT6 methylated by

PRMT1 for 0, 5, 10, 30, 60, 120, 180, 240 min and then H3 added; (10) PRMT1 alone incubated with H3.

(B) Scanned phosphorimage of the SDS gel.
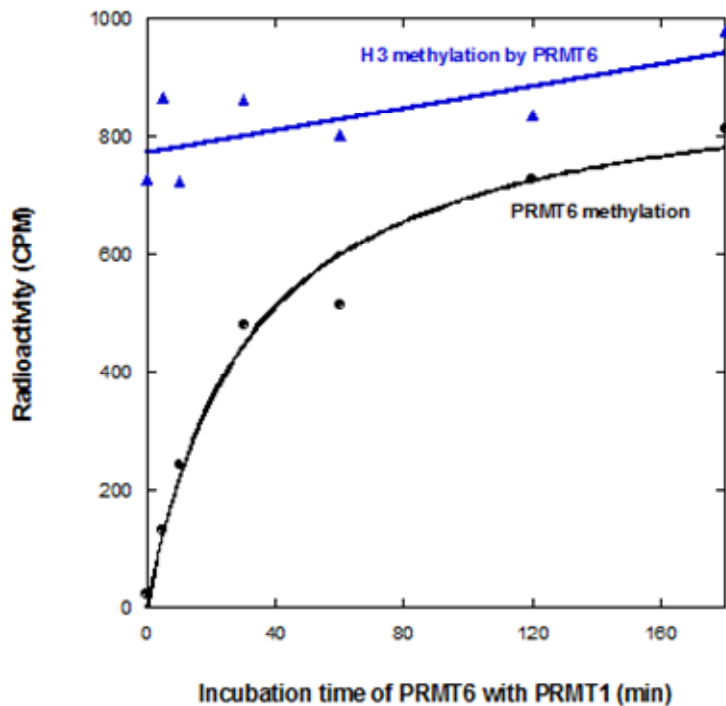
FigureS I-6. Rate graph of PRMT6 methylation of H3 Protein with increasing PRMT1
The phosphorimage in SI-5 was quantified to show PRMT6 methylation by PRMT1 with the introduction of H3 protein. The protein methylation is scattered but shows a gradual increase.
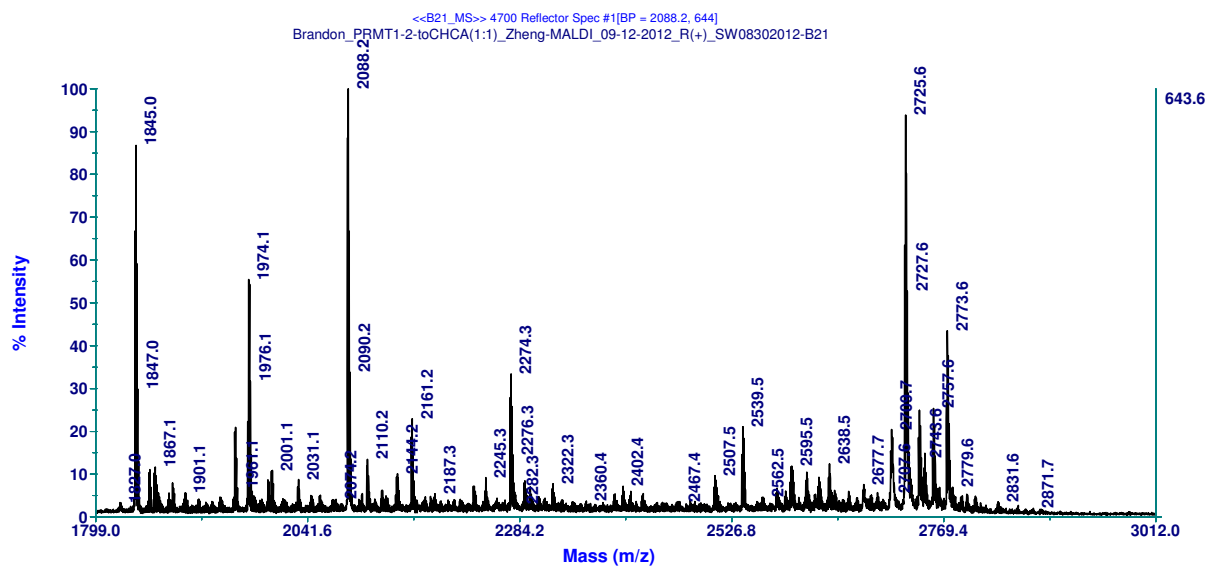
Figure SI-7. MALDI DMI-22 after HPLC purification

DMI-22 was not easily purified using RP-HPLC. The single peak observed contained a lot of impurities.
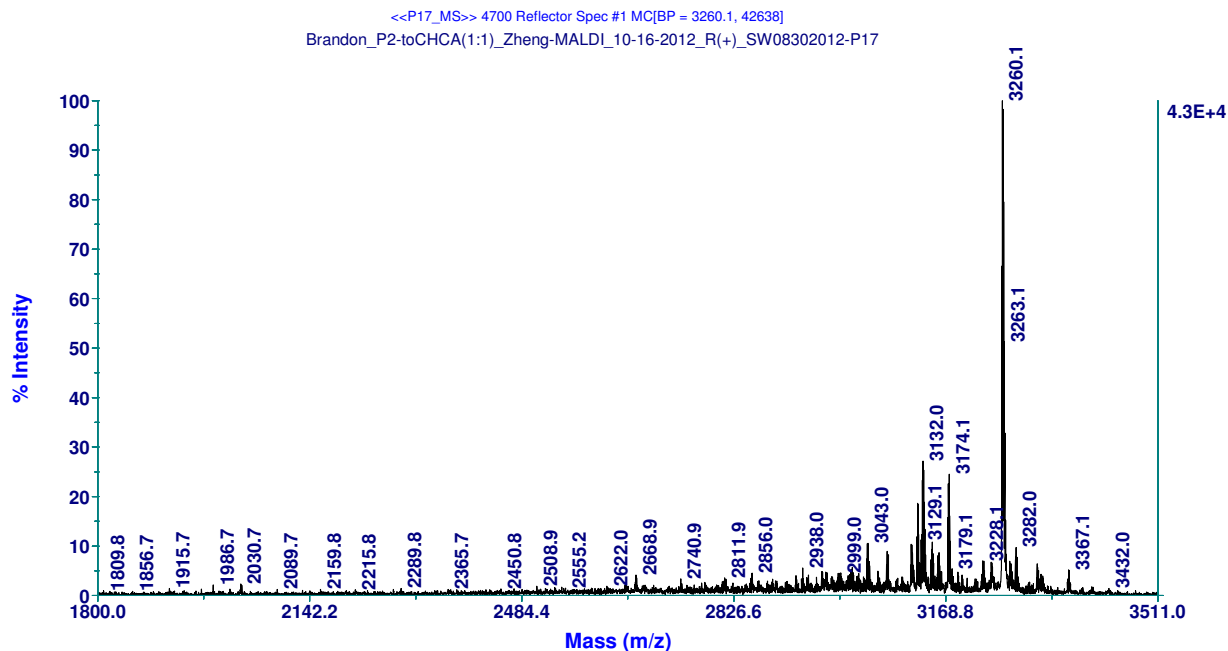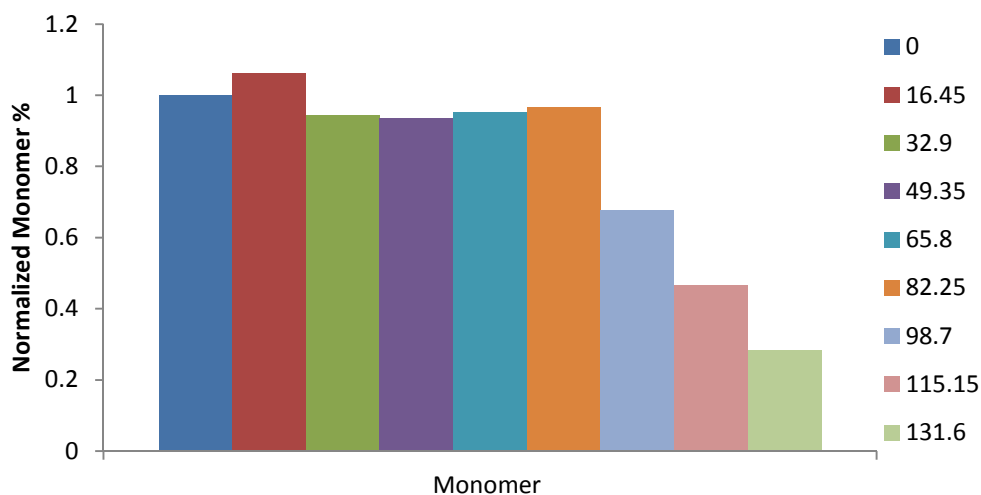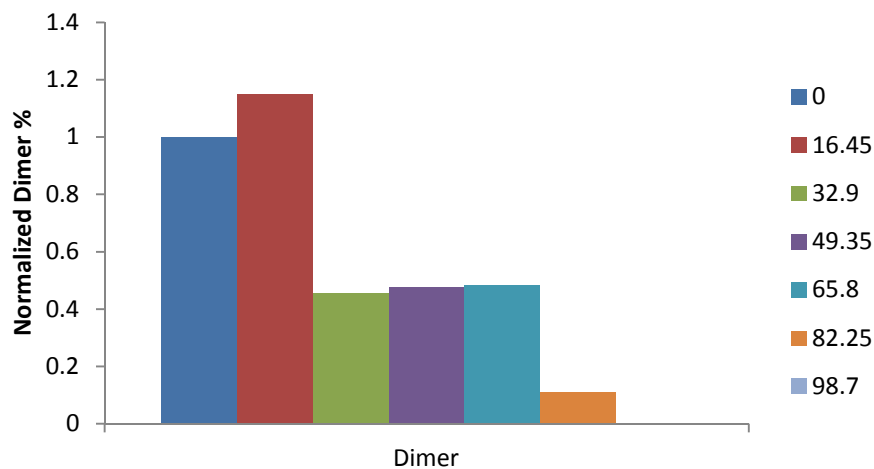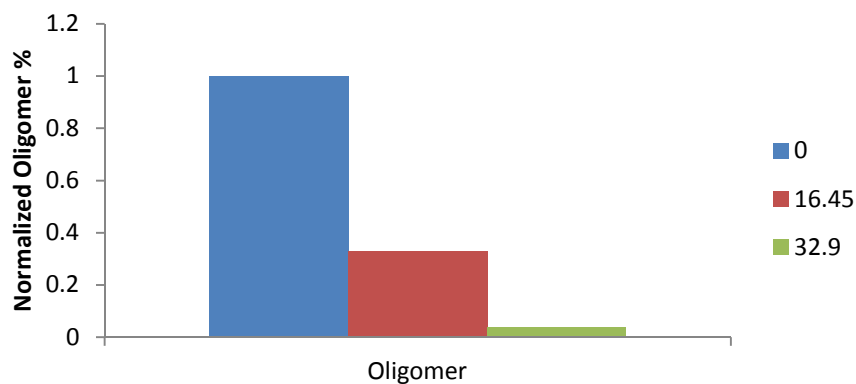

Figure SI-8. MALDI of DMI-26 after HPLC purification

DMI-26 was purified on the RP-HPLC. Impurities were removed and a relatively pure product was obtained after purification
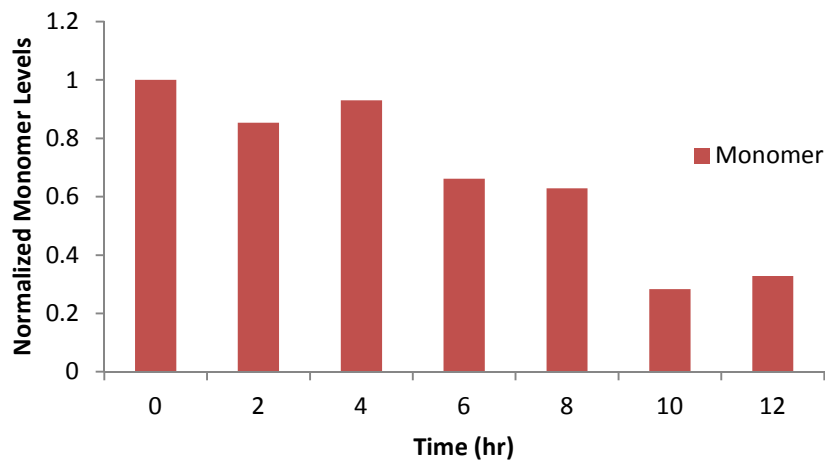
(A) Monomer analysis

(B)Dimer analysis

(C)Oligomer analysis

FigureS I-9. Western blot quantification for relative remaining PRMT1 states
The X-ray film from the gradient Western blot experiment was quantified with QuantityOne software to show the relative amounts of PRMT monomer, dimer, and oligomer remaining against the untreated PRMT1. The quantification was performed until the remaining state was >4%.

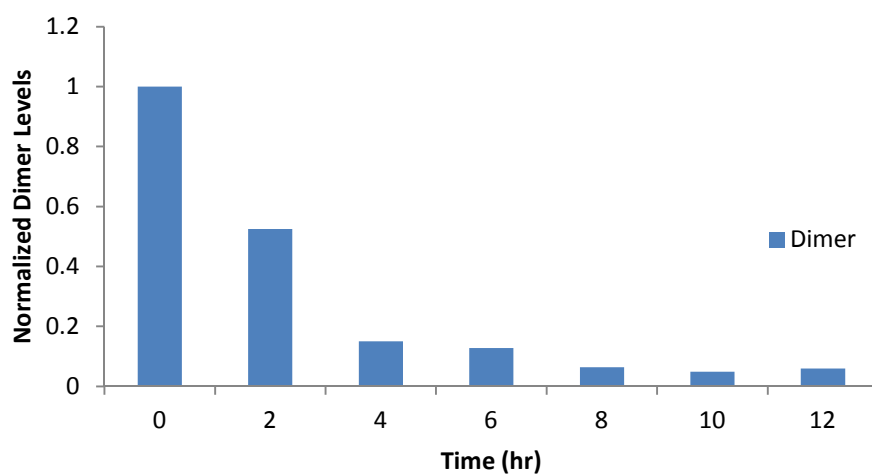**(A)Monomer analysis**

(B)Dimer analysis

Figure SI-10. Quantification of time based Western blot for PRMT1 and DMI
The X-ray film from the time based Western blot experiment was quantified with QuantityOne software to show the relative amounts of PRMT monomer and dimer remaining against the initial time point of PRMT1 and DMI-26 incubation. The quantification was performed from 0-12 hour on the X-ray film.
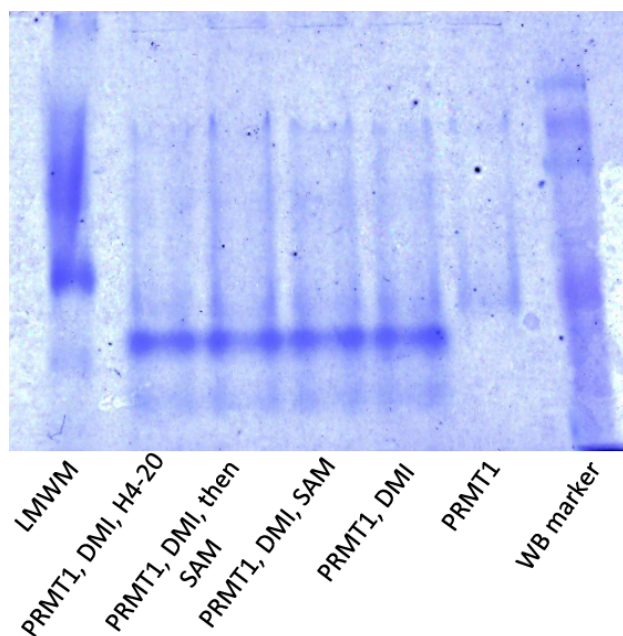
Figure SI-11. Native PAGE of PRMT1 with DMI, SAM, and/or H4(1-20)
The native gel was loaded with the samples from the Western blot. 20 μl of the incubation mixtures were loaded to each well. The 10% native gel was run at 100V for 5 hours. The gel shows no distinct PRMT1 band. The DMI-26 band can be observed. The markers contain SDS and smeared as the proteins were resolved.