İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IN VIVO METHYLATION STUDIES OF MEFV GENE

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MEFV GENİNİN İN VİVO METİLASYON ÇALIŞMALARI

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TABLE OF CONTENTS

ABBREVIATIONS

FMF : Familial Mediterranean Fever

MEFV : Mediterranean Fever (gene)

IL : Interleukin

PCR : Polymerase Chain Reaction

DNA : Deoxyribonucleic Acid

MSP :Metylation Specific PCR

BSS :Bisulfite Sequencing

M Primer :Methylated, bisulfate treated DNA-specific primer

U Primer :Unmethylated, bisulfate treated DNA-specific primer

LIST OF TABLES

LIST OF FIGURES

In vivo Methylation Studies of MEFV Gene

SUMMARY

MEFV gene was first identified as the candidate gene for Familial Mediterranean Fever (FMF),which proceed with serosal inflammation and abdominal pain. Most common MEFV mutations are E148Q, M680I, M694V, M694I, and V726A which account for the 70% of the disease chromosomes. Among these mutations, only E148Q was reported to be associated with a milder phenotype of the disease. E148Q mutation lies within the second exon of MEFV gene and causes a nucleotide substitution of Guanine to Cytosine at nucleotide position 442. E148Q mutation was reported to increase MEFV expression whereas other MEFV-related mutations were shown to decrease MEFV expression. The milder phenotype in the presence of E148Q mutation is thought to be due to its increasing effect on MEFV gene expression.

The electronical analysis of MEFV gene with bioinformatic tools, gives a CpG island spanning the second exon of MEFV gene. Moreover, within this CpG island, E148Q mutation causes the elimination of one CpG dinucleotide. This study is focused on the relationship between the methylation pattern of second exon of MEFV gene in relation with the E148Q mutation.

The techniques used to identify this DNA methylation pattern are Methylation Specific PCR (MSP) and Bisulfite Sequencing (BS). Methylation specific PCR was used to obtain a preliminary data of DNA methylation at the second exon of MEFV gene, whereas methylation quantification was performed by direct bisulfite sequencing.

Consequently, in this study, the methylation pattern of the second exon of MEFV gene was determined. This methylation pattern was compared between E148Q heterozygotes and wildtype controls as well as FMF samples and healthy controls. A significant decrease of CpG methylation at the E148Q site was observed in

heterozygotes compared to wildtype samples. No significant difference was found in the methylation pattern of the second exon of MEFV gene between FMF samples and healthy controls.

DNA methylation patterns of wildtype and E148Q heterozygote samples were also compared with MEFV expression levels determined by another study of human genetics group. Methylation level of the CpG dinucleotide at E148Q site found to be significantly associated with MEFV expression level.

In addition, DNA methylation of the second exon was compared between FMF and healthy samples. Within the CpG sites analyzed, no significant difference of DNA methylation was found at CpG dinucleotides between FMF samples and controls except that at three positions DNA methylation was 10-15% lower in FMF patients.

MEFV Geninin *in vivo* Metilasyon Çalışmaları

ÖZET

MEFV geni ilk olarak serozal iltihaplanma ve karın ağrısı ile ilerleyen Ailevi Akdeniz Ateşi (AAA) hastalığıyla ilişkili gen olarak karakterize edilmiştir. En sık gözlenen MEFV mutasyonları E148Q, M680I, M694V, M694I ve V726A olup hastalıkla ilgili kromozomların %70'ini oluştururlar. Bu mutasyonlar arasında yalnız E148Q mutasyonunun hastalığın daha hafif geçmesini sağlayıcı rolü olduğu bilinmektedir. E148Q mutasyonu MEFV geninin ikinci ekzonunda bulunmakta olup 442. pozisyondaki guanine nükleotidini sitozine çevirmektedir. Diğer MEFV mutasyonları MEFV gen ifadesini azaltırken sadece E148Q mutasyonunun MEFV gen ifadesini artırdığı bildirilmiştir. E148Q'ya bağlı gen ifadesi artışının FMF hastalığının daha hafif geçmesiyle ilişkili olduğu düşünülmektedir.

MEFV geninin elektronik ortamda biyoinformatik araçlarla analizi genin ikinci ekzonunu içine alan bir CpG adacığı olduğunu göstermektedir. Bununla birlikte bu CpG adacığının içinde yer alan E148Q mutasyonu bir CpG dinükleotidini ortadan kaldırmaktadır. Bu çalışma MEFV geninin ikinci ekzonunun metilasyon paterni ile E148Q mutasyonu arasındaki ilişkiye odaklanmıştır.

DNA metilasyon paterninin aydınlatılması için kullanılan teknikler metilasyona özel polimeraz zincir reaksiyonu (PZR) ve bisülfat dizilemesi (BD) yöntemleridir. Metilasyona özel PZR yöntemi MEFV geninin ikinci ekzonununun metilasyonu hakkında ön bilgi edinmek amacıyla yapılmış olup, bisülfat dizilemesi yoluyla da metilasyonun kantifikasyonu yapılmıştır.

Bu çalışmada sonuç olarak MEFV geninin ikinci ekzonunun metilasyon paterni belirlenmiştir. Bu metilasyon paterni E148Q heterozigotları ile yabanıl genotipe sahip gruplar arasında ve AAA hastaları ile sağlıklı kontroller arasında karşılaştırılmıştır. E148Q mutasyonunun denk geldiği CpG dinükleotidinde yabanıl genotipe kıyasla E148Q heterozigotlarında belirgin bir düşüş gözlenmiştir. Bununla

birlikte, AAA hastaları ile sağlıklı kontroller arasında MEFV geninin ikinci ekzonunun metilasyonu açısından kayda değer bir fark bulunamamıştır.

Yabanıl tip ve E148Q heterozigot örneklerin DNA metilasyon paterni bu örneklerin daha önce İnsan Genetiği grubu tarafından belirlenmiş MEFV gen ifadesi seviyeleri ile karşılaştırılmıştır. E148Q dinükleotidinin denk geldiği CpG adacığının metilasyon seviyesi ile MEFV gen ifadesi seviyeleri ile karşılaştırılmıştır. E148Q dinükleotidinin denk geldiği CpG adacığının metilasyon seviyesi ile MEFV gen ifadesi arasında önemli bir ilişki bulunmuştur.

Buna ek olarak, ikinci ekzonun metilasyonu FMF hastaları ve sağlıklı kontrol grubu arasında kıyaslanmıştır. İki grup arasında incelenen CpG bölgelerinde metilasyon açısından kayda değer bir fark bulunmamıştır ancak üç CpG bölgesinde DNA metilasyonu FMF hastalarında % 10-15 daha az olarak gözlenmiştir.

1. INTRODUCTION

1.1. THEORETICAL PART

1.1.1. FMF AND MEFV GENE

1.1.1.1. FAMILIAL MEDITERRENEAN FEVER DISEASE

Familial Mediterranean Fever (FMF) is classified as a member of hereditary periodic fever syndromes, which are also known as autoinflammatory disorders [1]. FMF isreported to be an autosomal recessive disorder commonly seen in people of Mediterranean origin including Armenians, Sephardi and Ashkenazi Jews as well as Turks and Arabs [2]. Typical symptoms include development of transient fever and inflammation inserosal membranes, accompanied by abdominal pain [3]. Untreated cases usually develop amyloidosis together with renal failure,which is the primary complication of FMFdisease [4]. Clinical diagnosis is based on these periodically seen attacks of inflammation and amyloidosis in more serious cases. In addition to attacks, MEFV mutations are usually screened to support diagnostic findings. Treatment of the diease is based on daily colchicine therapy. In majority of the cases treated with colchicine, it is observed that FMF attacks are reduced or completely prevented. It was also reported that colchicine therapy is preventive against FMF-related amyloidosis [2].

1.1.1.2. THE MEDITERRENEAN FEVER GENE, MEFV

MEFV gene was first characterized to be the candidate gene for Familial Mediterrenean Fever in 1997 by International FMF Consortium [5]. In the same year, French FMF Consortium reported that the nucleotide changes concentrated at the carboxy-terminal of the MEFV gene are responsible for FMF disease [6].

Spanning 16kb region on the human genome, MEFV gene is located on chromosome 16p, and is composed of 10 exons [7]. The gene codes for a 3.7kb transcript [5, 9] and expressed mainly in neutrophils, eosinophils, and cytokine activated monocytes

[8,9].MEFV expression was shown to be increased by activators of inflammation including Tumor Necrosis Factor Alpha and Interferon gamma, which led to speculations about an essential role of this gene in the inflammatory response [14]. In addition, CD34 positive hematopoietic stem cells were shown to be induced towards granulocytic lineage when MEFV was expressed at myelocyte stage, which assigns a role to MEFV genein granulocytic lineage specification [9].

1.1.1.3. STRUCTURE AND FUNCTION OF PYRIN, THE PROTEIN PRODUCT OF MEFV GENE

MEFV gene codes for a 781 aminoacid protein named pyrin / marenostrin [10]. Denomination was made on the basis of Latin and Greek words where pyrin means fever (in Greek) and marenostrin means Mediterranean Sea (in Latin) [11].

Marenostrin protein contains four domains, which are PYD domain (Pyrin Domain, N Terminal Domain), B30.2 domain (C Terminal Domain, PRYSPRY domain), the Bzıp transcription factor basic domain and B-box zinc finger coiled coil domain (Figure 1.1). Among these domains PYD (pyrin) domain is important to estimate the function of the pyrin protein. PYD domains are death domain like structures, which have immune system related functions [12].

After the prediction of secondary structure of pyrin domain by computational techniques, pyrin domain is proposed to be a member of a superfamily which contain caspase activation and recruitment domains (CARDs), death domains (DDs), and death effector domains (DEDs) [15].

Another domain of pyrin protein, B-box zinc finger coiled coil domain, is capable of making homodimers and heterodimers with other proteins, whereas the pyrin (PYD) domain makes only homodimers with other pyrin domains [15].

The PRYSPRY (B30.2) domain is a very common protein – protein interaction domain which is known to interact with approximately 500 different proteins. This domain is commonly found in proteins, which have immune system related functions like cytokine signaling. In pyrin protein, FMF related mutations are concentrated on this domain and nearly all of these mutations are reported to be on sites of flexible protein binding loops [15].

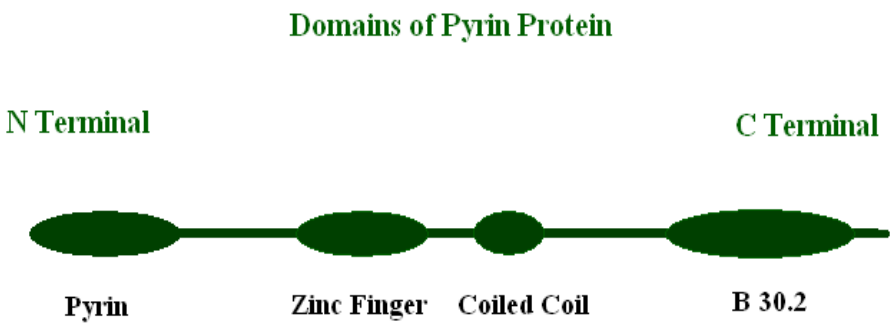

Figure 1.1 Domains of pyrin protein

Pyrin protein was shown to interact with a number of other PYD domain containing proteins via PYD/PYD domain interactions. The multi-PYD domain interactions are shown to be mediated by an adaptor protein named ASC (apoptosis-associated speck-like protein whichcontains a caspase recruitment domain) that has important roles on programmed cell death, pro-caspase 1 activation and IL-1beta processing as well as triggering NF-kappaB response [12]. Depending on these findings, pyrin is thought to be involved in inflammation and neutrophil apoptosis [12, 13]. Furthermore, Pyrin was suggested to be a negative regulator of granulocyte-mediated inflammation [15]. Although the mutant form of pyrin is thougt to prevent programmed cell death and cause high IL-1beta levels, which lead to inflammation (Figure 1.2), no difference was detected between the binding of mutated and wildtype pyrin by means of their association with ASC which indicate an alternative pathyway to ASC mediated pyrin function [2].

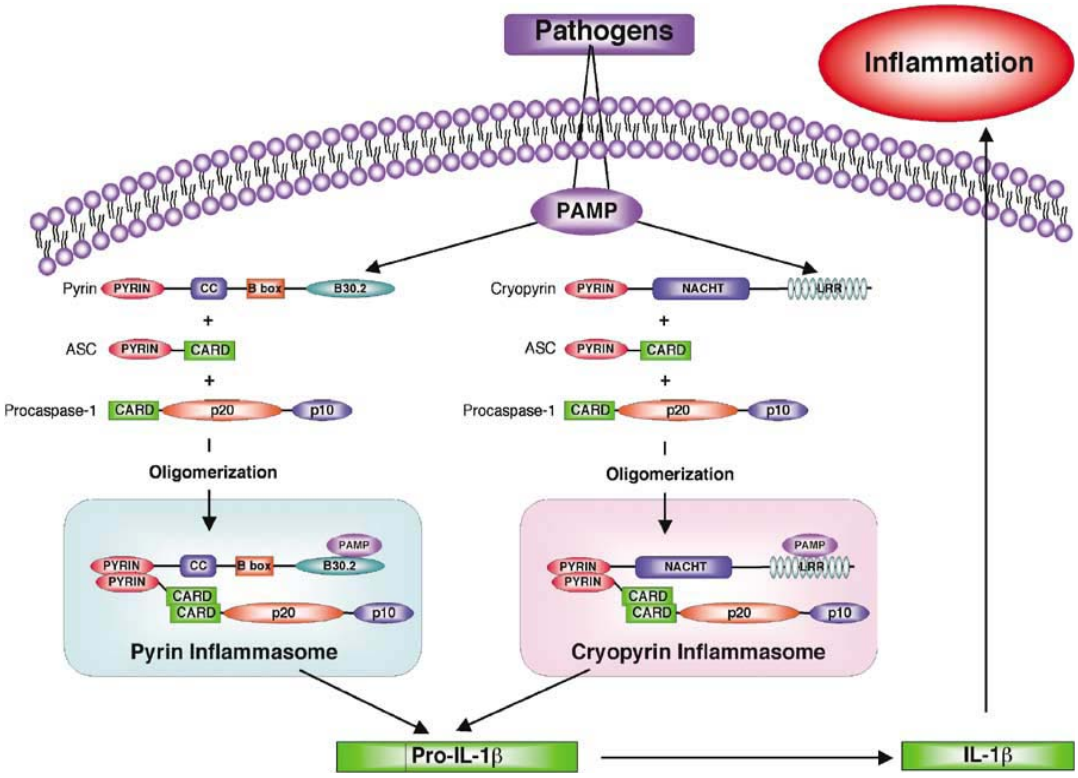

Figure 1.2 Estimated model of pyrin action in IL-1beta mediated inflammation [16]

In another study, Chae *et al.* created transgenic mice, which express mutant pyrin protein and observed lipopolysaccharide induced fever and inflammation. In homozygous state, the mutation was reported to increase lethality. Activated macrophages of these mice showed higher amounts of activated caspase 1 and IL-1beta (Figure 1.3)[19]. Thedata all together place pyrin protein in myelomonocytic-specific proinflammatory pathway triggered by lipopolysaccharides and some anti-inflammatory factors including specific type of interleukins [18].

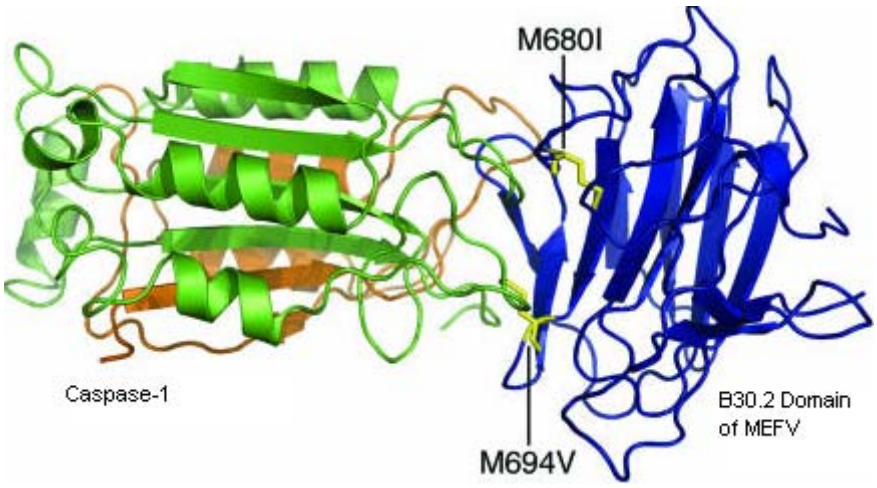

Figure 1.3 Proposed model of Pyrin B30.2 domain and Caspase-1 interaction [8]

Pyrin protein has two possible nuclear localization signalsin its sequence (amino acids 419-422, and 420-437) identified by bioinformatic softwares [17]. Besides these signals, pyrin shows homology to some regulators of transcription. Depending on these facts, Mansfield *et al.*proposed that pyrin may also be a regulator of transcription [14].

To date, one common alternatively spliced form of pyrin is defined, which lacks the second exon and causes an in-frame spliced form. Full lenth pyrin was shown to be localized in the entire cytoplasm whereas the spliced form is mainly concentrated in the nucleus. It was also shown that elimination of the two nuclear localization signals does not affect the nuclear localization of the spliced form suggesting an overall regulatory function of the lacking domain on the subcellular localization of pyrin [17].

In another study, the relationship between pyrin and colchicine responsiveness was studied. What makes FMF differ from other periodic fever syndromes is the colchicine responsiveness. Pyrin is thought to be involved in a colchicine – responsive inflammatory pathway. The colchicine action is not related to granulocyte activity at the inflammation site but increase in the FMF related inflammatory infiltrate. Low doses of colchicine does not affect the total length of microtubule but constricts interchange of tubulin dimers at microtubule ends whereas high levels of colchicine inhibits plus end polymerization. Colchicine is supposed to have a role on microtubule dynamics in granulocytes which leads to poor adhesion and migration capability [14]. In addition to this data, Pyrin was found to be associated with cytoskeletal elements including mictotubules and actin filaments by Mansfield *et al.*They have found a cytoplasmic localization of full length pyrin, and detected colocalizaiton of pyrin with actin and mictobule cytoskeleton within the cell. They have also shown that pyrin – microtubule interaction was provided via the N terminal domain (B30.2) of pyrin protein [14]. To investigate the colchicine responsiveness in FMF, further studies need to be done.

1.1.1.4. COMMON MEFV MUTATIONS AND MEFV EXPRESSION LEVELS

Nearly 30 sequence variants of MEFV gene was reported to be associated with FMF [20]. Five FMF mutations, M694V, V726A, M694I, M680I and E148Q correspondto aproximately 75% of FMF alleles [15]. Among severe number of MEFV mutations,

thisfivemutationsseem to be responsible for most of the FMF cases. Except from E148Q mutation, which was reported to cause a milder phenotype in FMF, other four mutations located in the 10th exon which correspond to PRYSPRY domain of pyrin protein [15].

The relationship between FMF disease and MEFV mutations remains to be clarified. Although FMF was reported to be an autosomal recessive disorder, there are non-symptomatic cases which carry two mutant alleles of MEFV (either homozygous for one mutation or compound heterozygotes). On the contrary, some FMF cases are reported not to have any MEFV mutations [2]. There are suggestions about the type of the mutation and FMF disease relation. The most commonly seen form of MEFV mutation among FMF patients is M694V, which is tought to cause more drastic symptoms in FMF with a ranging allele frequency among populations like 45% in Armenians [21], 52% in Turkish population [22], and 80% in Iraqi and North African Jewish population [23]. Located at the second exon of MEFV gene, E148Q mutation was reported to be most frequently seen sequence variant in general population (non-FMF subjects) [7]that is related with the milder phenotype in FMF [2].

To establish the relationship between certain MEFV mutations and FMF disease pathology, several gene expression studies were done. In 2002, Notarnicola *et al.* studied the relationship between MEFV expression levels of peripheral blood leukocytes and FMF. They reported that MEFV expression levels are lower in FMF patients compared to non-founder controls. According to their results, M694V mutation was related with considerably lower levels of MEFV expression. In addition, they have shown that the number of carrier M694V alleles are important in MEFV expression levels. Homozygote mutant cases show lower MEFV expression levels than heterozygotes. Besides, showing FMF symptoms is a factor that decreases MEFV expression among M694V mutation carriers [24]. In another study, Üstek *et al.* confirmed that MEFV expression is lower in FMF patients compared to non-founder control group, and further studied MEFV expression levels of peripheral blood leukocytes in FMF patients during asymptomatic period and FMF attacks. They have reported that MEFV expression levels were drastically decreased during attacks of FMF [25].On the other hand, patients carrying E148Q mutation showed higher MEFV expression than other FMF patients [24]. This data was consistent with the suggestions of Touitou (2001) about E148Q mutation and its protective effect in

FMF patients. In that study it was reported that 55% of the cases who are homozygous for E148Q did not show any disease symptoms and never developed amyloidosis [26]. Mimouni *et al.* also founded that among serious FMF cases, E148Q carriers did not developed amyloidosis [27]. In contrast with these E148Q carriers, compound heterozygotes with E148Q commonly show amyloidosis suggesting a more severe effect of this allele in compound heterozygote form [26].

Touitou proposed that the genotype phenotype correlation in FMF needs further study to be established, like exploration of complex alleles, modifier loci, genetic heterogenity as well as potential epigenetic regulators. In the case of E148Q mutation, causes of the phenotypic difference between E148Q carriers and compound heterozygotes remain to be established. It was suggested that some unknown genetic and epigenetic mechanisms may present behind E148Q case, and E148Q was defined as a functional polymorphism by this group [26].

1.1.2. DNA METHYLATION AND EPIGENETICS

1.1.2.1. EPIGENETIC MECHANISMS IN HIGHER EUKARYOTES

Epigenetics is defined as the non-genetic mechanisms, which has roles on gene expression. Epigenetic changes include histone modifications and DNA methylation [30]. Most commonly studied epigenetic mechanism is DNA methylation, which is involved in gene silencing in mammals [28]. DNA methylation is known to have effects on cellular mechanisms like development, X chromosome inactivation and imprinting, inhibition of foreign DNA, T cell action, aging, and memory [29].

CpG dinucleotides are not equally distributed throughout the human genome. Most of the CpG dinucleotides are concentrated at the promoter sites of genes. CpG clusters with a GC content of above 55% is termed as CpG islands. They are usually bigger than 0.5kb and have a minimum observed/expected CpG density of 0.65 [30].

DNA methylation occurs at the cytosine residues of CpG dinucleotides in the mammalian genome. Normally, 50 to 70 percent of CpG sites are methylated in the human genome [30].

DNA methylation is carried out by special enzymes called DNA metyhltransferases (DNMTs). DNMTs transfer the methyl group from S-adenosylmethione (SAM) to 5th carbon of cytosine nucleotide (Figure 1.4). Different types of DNMTs are

identified to date. DNMT1 is the maintanance methylase which is responsible for copying original methylation pattern of parental DNA to newly formed DNA during replication. Other methylases are DNMT3a and DNMT3b, which are identified as *de novo* methylases and responsible for methylation of unmethylated DNA usually in embryonic stem cells [30].

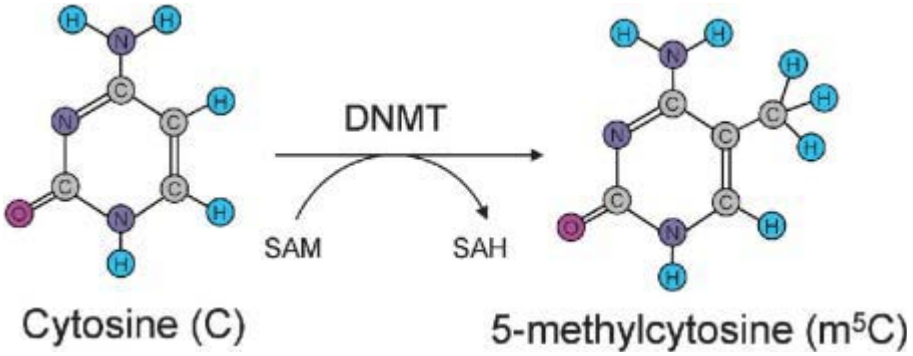

Figure 1.4 Cytosine methylation by DNA methyltransferases

In addition to DNA methylation, histone modifications play important roles in terms of epigenetic regulation. 5 types of histones (H2A, H2B, H3 and H4) come together to form the nucleosome structure, which is then surrounded by a 146 bp DNA. Modifications of these histone proteins like histone 3 lysine 4 trimethylation, histone 3 lysine 9 trimethylation, and histone acetylation at certain aminoacids trigger chromatin remodelling mechanisms, which is responsible for tight or loose packaging of chromatin and indeed causes gene expression changes (Figure 1.5) [30].

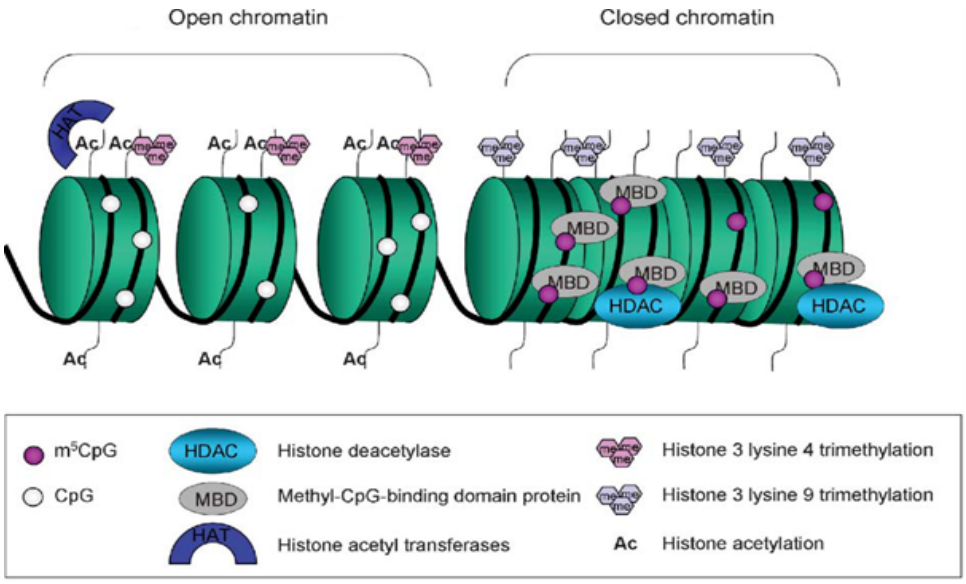

Figure 1.5 Histone modifications and chromatin state

When the methyl group is transferred from SAM, the resulted molecule is called S-adenosylhomocysteine (SAH), which is concerted to homocysteine by removal of adenosine part. For maintanance of SAM levels, SAH should be converted to methionine, which is the precursor of SAM. This process is closely related to folic acid, since it is needed for conversion of SAH to methionine. Elevated levels of homocysteine due to low folate intake was reported to be associated with more than five cancer types, cardiovascular diseases, cerebrovascular diseases, diabetes, and developmental as well as neurological disorders [29].

De novo methylation of cancer related genes including tumor suppressors are widely studied, but the effects of DNA methylation on aging and familial diseaseas remains to be established [28].

1.1.2.2. GENE EXPRESSION REGULATION VIA DNA METHYLATION

DNA methylation is a common transcriptional regulation mechanism in mammals. Effects of DNA methylation on gene expression was commonly studied in the promoters of some disease-associted genes including several types of cancers. To date, severe number of cases were shown about the effects of aberrant DNA methylation of promoters on disease-associated gene expression changes. For example, the increase of promoter methylation in tumor suppressor gene p53 was reported to be associated with several cancer types including hepatocellular carcinoma [32], breast cancer [33], and lung cancer [34]. In addition to promoter methylation, methylation status of intronal or exonal CpG islands are started to be studied and reported that these might be functional. In a study made by Babidge *et al.*, it was shown that in colorectal carcinomas, the second exon of the bcl-2 gene is aberrantly methylated [35]. In addition, Kempster *et al.* reported a methylation of the second exon of p16 gene is related with oesophageal cancer [36].

In addition to promoter methylation, transposable elements commonly contain methylated CpG sites which is often seen in intronic regions. Lorincz et al studied whether intragenic methylation has an effect on transcriptional efficiency and reported a slight decrease in constracts methylated in a region downstream of the promoter, compared to unmethylated construct. This finding shows that DNA methylation in the regions downstream of the promoters may also have an effect on transcriptional efficiency. The mechanism of intragenic methylation-related decrease

of transcriptional efficiency was reported to be related with altered chromatin structure at methylated sites [37].

In addition, methylation at the downstream sites of a promoter was shown to decrease Pol II density at the methylated sites, which indicate a decrease in the transcriptional elongation step; whereas there was no difference of PolII density at the promoter sites of methylated and unmethylated constructs which indicate that transcriptional initiation was not affected by DNA methylation at the sites downstream of the promoter. In short, methylated DNA at the downstream site of a promoter does not affect transcriptional initiation efficiency but decreases elongation efficiency due to the alteration in the state of the chromatin [37].

Moreover, DNA methylation in intragenic regions is began to be studied and significant associations was shown in different studies between intragenic methylation and gene expression. In one of these studies, Okitsu et al studied methylation of a luciferase transcription unit and reported that intragenic DNA methylation at this transcriptional unit is more effective on gene expression rather than promoter methylation. Shortly, intragenic DNA methylation can be as important as promoter methylation [38].

Besides intronal and exonal DNA methylations, there are also cases of allele specific changes in DNA methylation. Polesskaya *et al.* reported that allele-specific DNA methylation in 5HT2AR gene regulates the expression of this gene via establishing methylation difference between C and T alleles of a polymorphism in its first exon [39].

1.2. PRACTICAL PART

1.2.1. SNP GENOTYPING

Single nucleotide polymorphisms were first studied in 1980s by using restriction enzymes to discrminate between wildtype and polymorphic allele depending on the fragment length after restriction enzyme digestion [31].

1.2.1.1. POLYMERASE CHAIN REACTION

Polymerase chain reaction is a technique used for *in vitro* amplification of target DNA sequences. Short oligonucleotide primers are used to prime the DNA synthesis reaction by heat-stable DNA polymerases. Subsequent cycling od denaturation, annealing and extension steps by thermal cycling, target DNA sequence is logarithmically amplified. PCR is used in many applications in molecular biology including genotyping, cloning, mutagenesis, and DNA sequencing [40].

1.2.1.2. RESTRICTION ENZYME DIGESTION

Restriction endonucleases are special enymes, which are capable of recognizing specific sequences on DNA and cutting of that DNA [41]. Thesere are two types of restriction endonucleases called type I and type II restriction enzymes. Type I endonucleases are capable of cutting the DNA within their recognition site on the DNA, whereas type II endonucleases recognize a specific DNA sequence and cut the DNA at a neighbourhood site of that sequence.

Restriction endonucleases are used in several molecular biology applications including genotyping of single nucleotide polymorphisms where the SNP eliminates or introduces a restrciton site on the DNA. Depending on the type, these SNPs change the restriction fragment patterns and commonly used for detection of genotypes in molecular biology [42].

1.2.1.3. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a commonly used and highly effective technique to separate, identify, and purify DNA fragments usually within the range of 0.5- to 25-kb. The basic procedure in electrophoresis has three main stages, which are gel preparation with a determined agarose concentration useful to separate DNA fragments of wanted sizes, loading of DNA samples into the wells of the gel and running of the gel at a determined voltage and time period which will allow complete separation of DNA fragments and staining of the gel with ethidium bromide and visualization by illumination with UV light [43, 44].

1.2.2. DNA METHYLATION DETECTION

1.2.2.1. BISULFITE TREATMENT

The addition of methyl groups to cytosine nucleotide of DNA is a commonly studied epigenetic DNA marker. Most of the technologies used for DNA methylation analysis are based on a chemical reaction, named ‘bisulfite treatment’, which makes methylation-dependent nucleotide changes by selective chemical conversion of non-methylated cytosine to uracil. In bisulfite treatment technique; all cytosine bases, which are non-methylated are converted to uracil but all methylated cytosine bases remain as cytosines. Bisulfite treated DNA than can be analyzed by using severe number of techniques like methylation specific PCR or direct bisulfite sequencing [45].

1.2.2.2. METHYLATION SPECIFIC PCR

Methylation Specific PCR (MSP) is a technique that used to discriminate between methylated and unmethylated bisulfite treated DNAs. The technique is based on desiging two sets of primers (M and U primers) each specific to certain type of DNA (methylated or unmethylated, bisulfite treated). M primers amplify only methylated, bisulfite treated DNA but not unmethylated, bisulfite treated DNA; whereas U primers amplify only unmethylated, bisulfite treated DNA but not methylated, bisulfite treated DNA [46, 47].

1.2.2.3. BISULFITE SEQUENCING

Bisulfite sequencing is a technique based on the amplification of methylated, bisulfite treated and unmethylated, bisulfite treated DNA fragments together followed by sequencing. Bisulfite sequencing can be applied directly to amplified DNA fragments (direct bisulfite sequencing) or by cloning of the PCR product and screening colonies by sequencing which is based on a statistical manner [48].

1.3 AIM OF THE STUDY

The aim of this study was to investigate the methylation pattern of the second exon of MEFV gene. In addition, two subgroups (E148Q heterozygotes and wildtypes) are selected to compare the DNA methylation patterns of the second exon since E148Q mutation eliminates a CpG site within the second exon. Furthermore, DNA

methylation levels of the CpG dinucleotide at the E148Q site was compared between wildtypes and E148Q heterozygotes. Since E148Q was known to increase MEFV expression, the methylation data was also compared in relation with MEFV gene expression determined by another study in our group. For further investigation about FMF disease and DNA methylation, DNA methylation levels of FMF samples and healthy controls were compared in connection with MEFV expression levels in these two groups.

2. MATERIALS AND METHODS

2.1. MATERIALS AND LABORATORY EQUIPMENTS

2.1.1. EQUIPMENTS

The laboratory equipments used in this study are listed in Appendix A.

2.1.2. CHEMICALS, ENZYMES AND MARKERS

The chemicals, enyzmes and markers used in this study are given in Appendix B.

2.2. CpG ISLAND ANALYSIS OF MEFV GENE

MEFV genomic sequence was analyzed in terms of CpG island content by using CpG island searcher software of University of Southern California. An illustration of the program screen is given in figure 2.1.

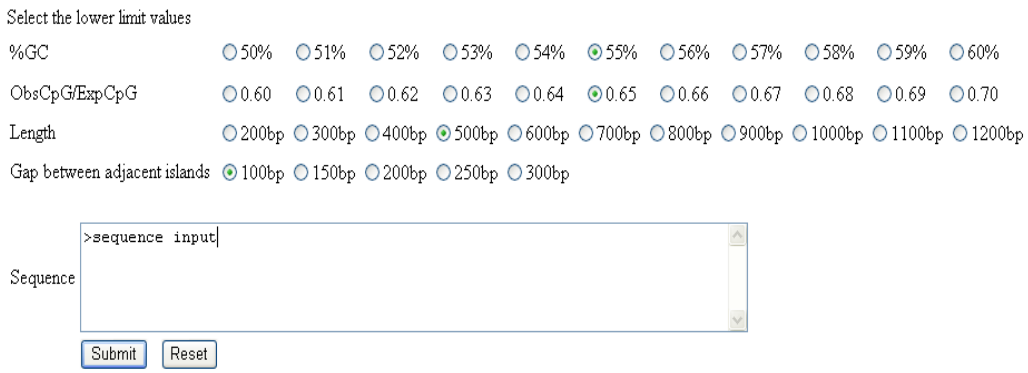

Figure 2.1: Illustration of CpG island software

2.3. DNA ISOLATION

2.3.1. COLLECTION AND STORAGE CONDITIONS OF BLOOD SAMPLES

Peripheral blood was obtained from people which come routine controls in Cerrahpaşa Medical Faculty Geriatrics Polyclinic. Blood samples were preserved in EDTA containing 5cc vacuum tubes, and stored at -20°C for short term and at -80°C for long term storage.

2.3.2. DNA ISOLATION FROM WHOLE BLOOD

The DNA isolation was performed by using Migration System 8Lx Instrument. DNA isolation kits were supplied by Precision System Science. DNA was isolated in an automated system and nearly 4 ml of blood was used for each sample. Isolated DNAs were kept at -20°C.

2.3.3. SPECTROPHOTOMETRIC CALCULATION OF DNA CONCENTRATIONS

Isolated genomic DNAs were diluted 1:100 ratio with sterile dH2O to a total volume of 500µl and absorbance values at 260nm, 280nm, and 320nm were measured. Concentration and DNA quality calculations from absorbance values are done according to the equations 2.1 and 2.2.

DNA Concentration (ng/µl) = (A260 - A320) x Dilution Factor (2.1)
DNA Quality = (A260 – A320) / (A280 – A320) (2.2)

2.3.4. PREPARATION OF WORKING ALIQUOTS OF GENOMIC DNA

To prepare working aliquots of 50ng/µl genomic DNAs, stock DNAs were diluted by using the equation 2.3.

M1xV1=M2xV2 (2.3)

Where,
M1=Stock DNA Concentration
V1=Volume of DNA gotten from Stock
M2=Aliquot DNA Concentration (50ng/µl)
V2=Final aliquot volume (500 µl)

2.4. E148Q GENOTYPING

2.4.1. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) was used to amplify the corresponding sequence within the second exon of MEFV gene which contain E148Q site. For PCR amplification, 50ng/µl working aliquots of genomic DNA was used as a template. To amplify 244 bp region containing E148Q site two oligonucleotide primers were used which are given in table 2.1.

Table 2.1: E148Q genotyping primers

Forward Primer	5'-ATATTCCACACAAGAAAACGGC-3'
Reverse Primer	5'-GCTTGCCCTGCGCG-3’

PCR mix used for the amplification of 244bp DNA fragment containing E148Q site is given in table 2.2.

Table 2.2. E148Q PCR mix

Stock Conc.	PCR Ing.	1X	Final Conc.
10X	Taq Buffer with KCl without Mg	2 µL	1X
2 mM	dNTP Mix	0.4 µL	40 µM
10 µM	Forward Primer	1 µL	0.5 µM
10 µM	Reverse Primer	1 µL	0.5 µM
50ng/µL	Genomic DNA	2 µL	5 ng/µL
5u/µL	Taq DNA Polymerase	0.2 µL	0.05 U/µL
25 mM	MgCl2	1.5 µL	1.875 mM
5X	Q Solution	4 µL	1X
	dH2O	7.9 µL	
	TOTAL	20 µL	

PCR program of E148Q genotyping PCR is given in table 2.3.

Table 2.3: Program of E148Q PCR

Initial Denaturation	96°C	5 min	
Denaturation	96°C	30 sec	X 5
Annealing	64°C	30 sec	
Extension	72°C	30 sec	
Denaturation	96°C	30 sec	X 35
Annealing	62°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	10 min	
Final Hold	4°C	∞	

2.4.2. AGAROSE JEL ELECTROPHORESIS FOR DETECTION OF PCR AMPLIFICATION

The expected DNA fragment length after PCR amplification was about 250 bp which is estimated to be clearly detectible in 1% agarose gel. To prepare 1% agarose gel in a mini electrophoresis system, 0.5 g agarose was dissolved in 50 µl 1X TBE buffer. 0.5 µg/mL ethidium bromide concentration was used for the gel. For each sample, 5 µl of PCR amplicon was mixed by 1 µl of 6X loading dye (Fermentas). As a

standard, Gene Ruler 1 kb DNA Ladder (Fermentas) was used to estimate the PCR amplicon sizes. Electrophoresis conditions were 40 minutes at 120V (12V/cm of gel). Pictures of the gels were taken under UV light with a transilluminator, by the help of UV PhotoMW software.

2.4.3. RESTRICTION ENZYME DIGESTION OF PCR PRODUCTS

Because the E148Q mutation eliminates a new AvaI restriction site from MEFV nucleotide sequence, PCR amplicons were digested with AvaI restriction endonuclease to determine the genoype of each DNA sample. Restriction digestion mix was prepared according to the table 2.4.

Table 2.4: Restriction mix for AvaI digestion

Ingredients	Amount	Final Concentration
10X Reaction Buffer	2 µl	1X
AvaI Restriction Enzyme (10U/µl)	1 µl	0.5U/µl
PCR Amplicon	12 µl	-
dH2O	5 µl	-
TOTAL	20 µl	

Restriction enzyme digestion was performed at 37°C for overnight followed by 15 min incubation at 80°C for restriction enzyme inactivation. The expected AvaI restriction digestion pattern of 244bp PCR amplicon is given in table 2.5.

Table 2.5: Restriction fragments of E148Q alleles

Wild Type	3 fragments (92+83+69 bp)
Mutant	2 fragments (161+83 bp)

2.4.4. DETERMINATION OF RESTRICTION PATTERN BY AGAROSE GEL ELECTROPHORESIS

Overnight digested samples were run on 3% agarose gel. 12µl of each sample was mixed with 2µl of 6X loading dye and loaded to the gel together with Gene Ruler Low Range DNA Ladder (Fermentas). Agarose gel was run at 100V for 1 hour for appropriate separation of digested DNA fragments. The gel pictures are taken by using UV PhotoMW software.

2.4.5. DETERMINATION OF GENOTYPES

Analysis of restriction pattern for all samples are done by comparing each restriction pattern according to the electronic digestion pattern shown below:

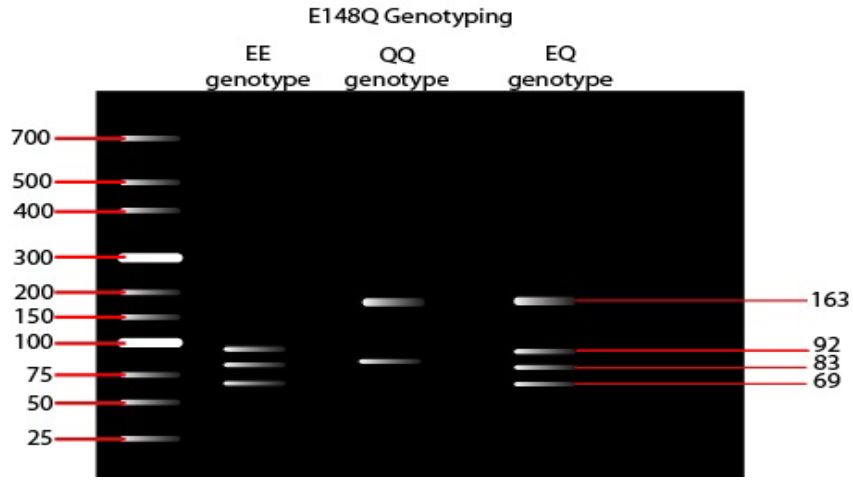

Figure 2.2: Expected restriction patterns of E148Q genotypes

2.5. DNA METHYLATION ANALYSIS

2.5.1. BISULFITE TREATMENT

For the analysis of DNA methylation patterns of 27 DNA samples (15 wildtype, 3 E148Q heterozygotes and 9 FMF samples) bisulfite treatment technique was used for chemical conversion of unmethylated cytosines to uracile nucleotide by deamination whereas methylated cytosines are not converted. Consequently, bisulfite treatments leads to two different DNAs for methylated and unmethylated forms of the same DNA fragment.

Since the initial DNA concentration used in this technique is limited because of the sensitive parameters of bisulfite conversion efficiency, 1µg of each DNA sample was used for bisulfite conversion (The supplier's recommended DNA amount is 1ng to 2µg). QIAGEN EpiTect bisulfite kit was used to perform bisulfite conversion. Main steps of the protocol include sodium bisulfite conversion of unmethylated cytosines, binding of the DNA to EpiTect column, washing and desulfonation steps, and finally elution of bisulfite converted DNA. The detailed protocol is given below:

Before starting to bisulfite reaction,

- 30 ml ethanol (96–100%) was added to Buffer BW and stored at room temperature (15–25°C). The bottle was inverted several times before starting the procedure.

- 27 ml of Ethanol (96–100%) was added to Buffer BD and stored at room temperature. The bottle was inverted several times before starting the procedure.
- 310 µl RNase-free water was added into the lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution. The carrier RNA was dissolved thoroughly by vortexing. Dissolved carrier RNA was added to Buffer BL.

Bisulfite Conversion Protocol:

- DNA to be used in the bisulfite reaction was thawn in room temperature.
- Bisulfite Mix was dissolved by adding 800 µl RNase-free water to each aliquot. Tubes are vortexed until the Bisulfite Mix is completely dissolved.
- Bisulfite reactions in 200 µl PCR tubes were prepared according to the table 2.6.

Table 2.6: Bisulfite reaction mix

Bisulfite Reaction Components	Component Volume per reaction (µl)
DNA solution	20 (1µg)
RNase-free water	-
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total volume	140

- PCR tubes are closed and the bisulfite reactions are mixed thoroughly.
- Bisulfite DNA conversion was performed using a thermal cycler according to the program given in table 2.7.

Table 2.7: Bisulfite reaction program

Step	Time	Temperature
Denaturation	5 min	99°C
Incubation	25 min	60°C
Denaturation	5 min	99°C
Incubation	85 min	60°C
Denaturation	5 min	99°C
Incubation	175 min	60°C
Hold	hold	20°C

- PCR tubes containing the bisulfite reactions were placed into the thermal cycler. Incubation was started with the program above.
- After the bisulfite conversion is complete, PCR tubes containing the bisulfite reactions were centrifuged briefly, and the complete bisulfite reactions were transferred to clean 1.5 ml microcentrifuge tubes.

- 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA was added onto the bisulfite reactions. The solutions are mixed by vortexing and centrifuge briefly.
- EpiTect spin columns are placed on collection tubes. The whole mixture from previous step was transferred into the EpiTect spin column.
- The column was centrifuged at maximum speed for 1 minute. Flow-through was discarded, and the spin column was placed back into the collection tube.
- 500 µl Buffer BW (wash buffer) was added into the spin column, and centrifuged at maximum speed for 1 min. The flow-through was discarded, and the spin column was placed back into the collection tube.
- 500 µl Buffer BD (desulfonation buffer) was added into the spin column, and incubated for 15 min at room temperature.
- The column was centrifuged at maximum speed for 1 min. The flow-through was discarded, and the spin column was placed back into the collection tube.
- 500 µl Buffer BW was added and column was centrifuged at maximum speed for 1 min. The flow-through was discarded, and the spin column was placed back into the collection tube. This step was repeated two times.
- The spin column was placed into a new 2 ml collection tube, and centrifuged at maximum speed for 1 min to remove any residual liquid.
- The spin column was placed into a clean 1.5 ml microcentrifuge tube. 20 µl Buffer EB was added to the center of the membrane. The purified DNA was eluted by centrifugation for 1 min at approximately 15,000 x *g* (12,000 rpm).
- Bisulfite treated DNAs were stored at –20°C.

2.5.2. METHYLATION SPECIFIC PCR

Methylation specific PCR (MSP) is a technique based on the amplification of bisulfite modified DNA with methylation specific primers. During the entire study, methylated, bisulfite treated DNA-specific primers are named as M primers whereas unmethylated, bisulfite treated DNA-specific primers are named as U primers. MSP needs two positive control DNAs which are the fully-methylated DNA and unmethylated DNA. MSP should be optimized for M and U primers such that M primers should only amplify methylated-bisulfite treated DNA but not the unmethylated-bisulfite treated DNA. Besides, U primers should amplify only

unmethylated, bisulfite treated DNA but not methylated, bisulfite treated DNA. These conditions are provided by using positive control DNAs and setting the stringency of PCR conditions.

2.5.2.1. AMPLIFICATION OF CONTROL DNA FRAGMENT FROM GENOMIC DNA FOR PREPARATION OF UNMETHYLATED MSP CONTROL

To prepare positive control DNA fragments for MSP, two primers were used to amplify a 567 bp region including the CpG island spanning the second exon of MEFV gene. Human genomic DNA was used as a template. The primer sequences used to amplify control DNA fragment is given in table 2.8. Primer locations on MEFV genomic sequence are given in figure 2.3.

Table 2.8: Primers used to amplify control DNA fragment

Primer	Size	Tm	GC%	Sequence	Product
MSPkF	21	**52.6**	42.8	5'-GCTTCATCATTTTGCATCTGG -3'	
MSPkR	20	**54.3**	50.0	5'-GATTCGCAGCTGTCTTTTCC -3'	**567 bp**

ctcaggtgatcctcccgcctcggcctcccagagtgccgggaatacaggca
tgagccaccgcgcccggcccgttgttttcctcaatttctaaactttaata
tccaaggggattctctctcctctgccctgaatcttgggccctaaacgtgg
gaca**gcttcatcattttgcatctgg**ttgtccttccagAATATTCCACACA
AGAAAACGGCACAGATGATTCCGCAGCGTCCAGCTCCCTGGGGGAGAACA
AGCCCAGGAGCCTGAAGACTCCAGACCACCCCGAGGGGAACGAGGGGAAC
GGCCCTCGGCCGTACGGGGGCGGAGCTGCCAGCCTGCGGTGCAGCCAGCC
CGAGGCCGGGAGGGGGCTGTCGAGGAAGCCCCTGAGCAAACGCAGAGAGA
AGGCCTCGGAGGGCCTGGACGCGCAGGGCAAGCCTCGGACCCGGAGCCCG
GCCCTGCCGGGCGGGAGAAGCCCCGGCCCCTGCAGGGCGCTAGAGGGGGG
CCAGGCCGAGGTCCGGCTGCGCAGAAACGCCAGCTCCGCGGGGAGGCTGC
AGGGGCTGGCGGGGGGCGCCCCGGGGCAGAAGGAGTGCAGGCCCTTCGAA
GTGTACCTGCCCTCGGGAAAGATGCGACCTAGAAGCCTTGAGGTCACCAT
TTCTACAGGGGAGAAGGCGCCCGCAAATCCAGAAATTCTCCTGACTCTAG
A**GGAAAAGACAGCTGCGAATC**TGGACTCGGCAACAGAACCCCGGGCAAGG
CCCACTCCGGATGGAGGGGCATCTGCGGACCTGAAGGAAGGCCCTGGAAA
TCCAGAACATTCGGTCACCGgtaaattgtgttctttcctactttatatcg
Gctgcagagaaagaatggctggccgggcacgatagctcatgcctgtaatc

Figure 2.3: Control primer sites on mefv genomic sequence. Upper cases indicate exon 2 of MEFV gene whereas lower cases show introns. Underlined nucleotides indicate control fragment primer sites.

PCR mix contents and PCR program used for amplification of control fragment are given in tables 2.9 and 2.10.

Table 2.9: PCR mix used for amplification of MSP control fragment

Stock Conc.	PCR Ing.	X	Final Conc.
10X	Taq Buffer with KCl and Mg	2.0µL	1X
10mM	dNTP Mix	0.4µL	0.4mM
10 µM	MSPkF	0.5µL	0.2 µM
10 µM	MSPkR	0.5µL	0.2 µM
100ng/µL	Human Genomic DNA	1µL	4ng/µL
5u/µL	Taq DNA Polymerase	0.2µL	0.1u/µL
-	dH2O	15.4	
	TOTAL	20 µL	

Table 2.10: PCR program used for amplification of MSP control fragment

Initial Denaturation	94°C	2 min	
Denaturation	94°C	30 sec	X40
Annealing	50°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	10 min	
Final Hold	4°C	∞	

2.5.2.2. PURIFICATION OF PCR PRODUCT

The PCR Product was purified bu using QIAGEN Gel Extraction Kit for further methylation reactions. PCR purification protocol is given below:

- 3 volumes of Buffer QG was added to 1 volume of the PCR sample and mixed.
- QIAquick spin column was placed in a provided 2 ml collection tube.
- To bind DNA, the sample was placed to the QIAquick column and centrifuged for 60 seconds.
- Flow-through was discarded and QIAquick column placed back into the same tube.
- To wash, 0.75 ml of Buffer PE was added to the QIAquick column and centrifuged for 60 seconds.
- Flow-through was discarded and the QIAquick column was placed back into the same tube.
- The column was centrifuged for an additional 1 min.
- QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
- To elute DNA, 50 µl Buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 minute.
- Eluted DNA was further used as unmethylated control for MSP, and also used to prepare methylated control for MSP.

2.5.2.3. SssI METHYLATION OF CONTROL FRAGMENT FOR PREPARATION OF METHYLATED MSP CONTROL

Preparation of methylated control DNA fragment was performed by SssI methylation of previously prepared unmethylated control fragment. 20 µl of unmethylated DNA fragment was methylated according to the following protocol on table 2.11.

Table 2.11: SssI Methylation mix

Ingredients:	Amount
10X NEB Buffer 2	5 µL
200X SAM	2 µL
SssI Methylase	1 µL
DNA (Control Fragment)	20 µL
dH2O	22 µL
Total	50 µL

The reaction mix was prepared and incubated at 37C for 3 hours. After 1,5 hour, additional 1 µL of SAM was added into the reaction mix. The final reaction included 30X SAM.

2.5.2.4. PURIFICATION OF METHYLATED DNA FRAGMENT

The PCR Product was purified bu using QIAGEN Gel Extraction Kit in order to obtain efficient bisulphite conversion in subsequent steps.

2.5.2.5. CONFIRMATION OF METHYLATION:

5 µL of methylated and 5 µL of unmethylated PCR products were digested by BstUI for 1 hour. Digestion products are run in 1% agarose gel in 1X TAE buffer.

2.5.2.6. BISULPHITE TREATMENT OF CONTROL FRAGMENTS

Both methylated and unmethylated control fragments were subjected to bisulphite treatment by using QIAGEN EpiTect bisulfite kit with the protocol given in methods.

2.5.2.7. MSP PRIMER DESIGN

There are several bioinformatic programs which can be used for designing MSP primers. In this study, MethylPrimer Express software was used to select M and U primers specific to a short part of the CpG site in the second exon of MEFV gene. After uploading target genomic DNA sequence, the software converts the genomic DNA to the bisulfite treated form by considering both the methylated and unmethylated forms of the target DNA fragment. Then, it chooses forward and

reverse primers on the regions which show most difference between bisulfite treated methylated and bisulfite treated unmethylated DNAs (Figure 2.4). These mostly different sites are the densely CpG containing sites. Each CpG site correspons to one base difference between M and U primers (Cytosine in the M primer and Uracil in the U primer, both in the case of forward primers). So, MSP primer selection criteria is that they should be selected at CpG containing sites to discriminate between methylated and unmethylated DNAs during PCR amplification.

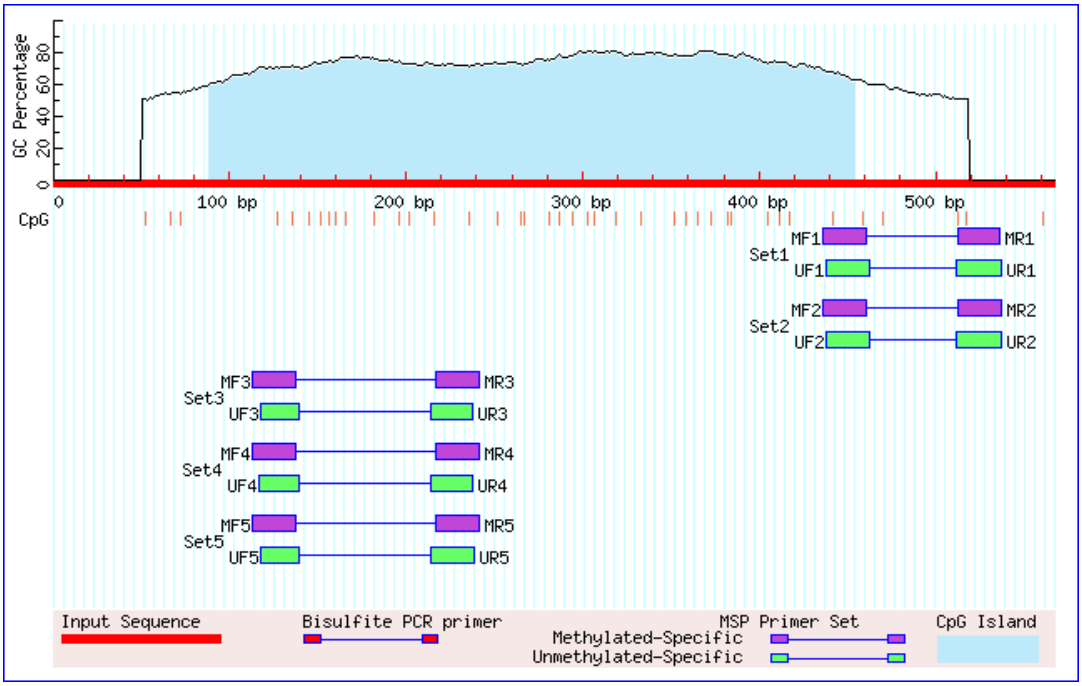

Figure 2.4: Methylation Specific Primer areas selected by MethylPrimer Express software

U primer sequence is given in table 2.12 whereas primer sites are shown in figure 2.5.

Table 2.12: MSP U primer sequences

Primer	Sequence	Product Size
Left 10 U	5'-TATAAGAAAATGGTATAGATGATTTTGT-3'	
Right 10 U	5'-CTCTACATTTACTCAAAAACTTCCTCAA-3'	201 bp

tttaggtgatttttttgttttggtttttagagtgttgggaatataggta
tgagttattgtgtttggtttgttgtttttttaatttttaaatttttaata
tttaaggggatttttttttttttgttttgaattttgggttttaaatgtgg
gatagttttattattttgtatttggttgttttttagAATATTTTA**TATA**
AGAAAATGGTATAGATGATTTTGTAGTGTTTAGTTTTTTGGGGGAGAATA
AGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATGAGGGGAAT
GGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTT
TGAGGTTGGGAGGGGGTTG**TTGAGGAAGTTTTTGAGTAAATGTAGAG**AGA
AGGTTTTGGAGGGTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTG
GTTTTGTTGGGTGGGAGAAGTTTTGGTTTTTGTAGGGTGTTAGAGGGGGG
TTAGGTTGAGGTTTGGTTGTGTAGAAATGTTAGTTTTGTGGGGAGGTTGT
AGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGTTTTTTGAA
GTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTAT
TTTTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAG
AGGAAAAGATAGTTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGG
TTTATTTTGGATGGAGGGGTATTTGTGGATTTGAAGGAAGGTTTTGGAAA
TTTAGAATATTTGGTTATTGgtaaattgtgttttttttattttatattg
Gttgtagagaaagaatggttggttgggtatgatagtttatgtttgtaatt

Figure 2.5: MSP U primers on unmethylated, bisulfite treated MEFV genomic DNA. Upper cases indicate exon 2 of MEFV gene whereas lower cases show introns

M primer sequence is given in table 2.13 whereas primer sites are shown in figure 2.6.

Table 2.13: MSP M primer sequences

Primer	Sequence	Product Size
Left 13 M	5'-AAGAAAACGGTATAGATGATTTCGT-3'	
Right 13 M	5'-CTACGTTTACTCAAAAACTTCCTCG-3'	**196 bp**

tttaggtgatttttttcgtttcggtttttagagtgtcgggaatataggta
tgagttatcgcgttcggttcgttgtttttttaatttttaaatttttaata
tttaaggggatttttttttttttgttttgaattttgggttttaaacgtgg
gatagttttattattttgtatttggttgttttttagAATATTTTATAT**A**
AGAAAACGGTATAGATGATTTCGTAGCGTTTAGTTTTTTGGGGGAGAATA
AGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACGAGGGGAA**C**
GGTTTT**C**GGT**C**GTA**C**GGGGG**C**GGAGTTGTTAGTTTGCGGTGTAGTTAGTT
CGAGGTCGGGAGGGGGTTGT**CGAGGAAGTTTTTGAGTAAACGTAG**AGAGA
AGGTTTCGGAGGGTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCG
GTTTTGTCGGGCGGGAGAAGTTTCGGTTTTTGTAGGGCGTTAGAGGGGGG
TTAGGTCGAGGTTCGGTTGCGTAGAAACGTTAGTTTCGCGGGGAGGTTGT
AGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGTTTTTCGAA
GTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTAT
TTTTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAG
AGGAAAAGATAGTTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGG
TTTATTTCGGATGGAGGGGTATTTGCGGATTTGAAGGAAGGTTTTGGAAA
TTTAGAATATTCGGTTATCGgtaaattgtgttttttttattttatatcg
Gttgtagagaaagaatggttggtcgggtacgatagtttatgtttgtaatt

Figure 2.6: MSP M primers on methylated, bisulfite treated MEFV genomic DNA. Upper cases indicate exon 2 of MEFV gene whereas lower cases show introns.

2.5.2.8. MSP PCR CONDITIONS

Methylation specific PCR was performed with the following protocol and program (Tables 2.13 and 2.14) by using hot start taq DNA polymerase.

Table 2.13: MSP PCR mix

Stock Conc.	PCR Ing.	1X	Final Conc.
10X	Hot Start Taq Buffer	2.0µL	1X
10mM	dNTP Mix	0.5µL	0.4mM
10 µM	Forward Primer	1.0µL	0.2 µM
10 µM	Reverse Primer	1.0µL	0.2 µM
100ng/µL	Template DNA	1.0µL	10ng/ul
5u/µL	Hot star Taq	0.2µL	0.1u/µL
-	dH2O	14.3	
	TOTAL	20 µL	

Table 2.14: MSP PCR program

Initial Denaturation	95°C	15 min	
Denaturation	95°C	30 sec	X35
Annealing	62°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	10 min	
Final Hold	4°C	∞	

PCR products were run on 1% agarose gel using 1X TAE buffer.

2.5.3. BISULFITE SEQUENCING (BSS)

Bisulfite sequencing is used to determine the exact methylation pattern of the second exon of MEFV gene. BSS is more informative compared to MSP technique since BSS allow quantification of DNA methylation at each CpG site. For BSS, two primers are designed to amplify bisulfite treated DNA, and since the primers are equally specific to methylated, bisulfite treated DNA and unmethylated, bisulfite treated DNA amplification of these two DNAs were performed within the same reaction. After amplification of target region, PCR products were sequenced.

2.5.3.1. BSS PRIMER DESIGN

To select appropriate BSS primers, MethPrimer software was used. The software chooses forward and reverse primers on the regions which show least difference

between bisulfite treated methylated and bisulfite treated unmethylated DNAs (Figure 2.7). These mostly different sites are the non-CpG sites. In contrast to MSP primers, BSS primers are common for both bisulfite treated methylated and bisulfite treated unmethylated DNAs. So, BSS primer selection criteria is that they should be selected at non-CpG sites to amplify methylated and unmethylated DNAs together with a common primer pair during PCR amplification. BSS primer sequences are given in table 2.15.

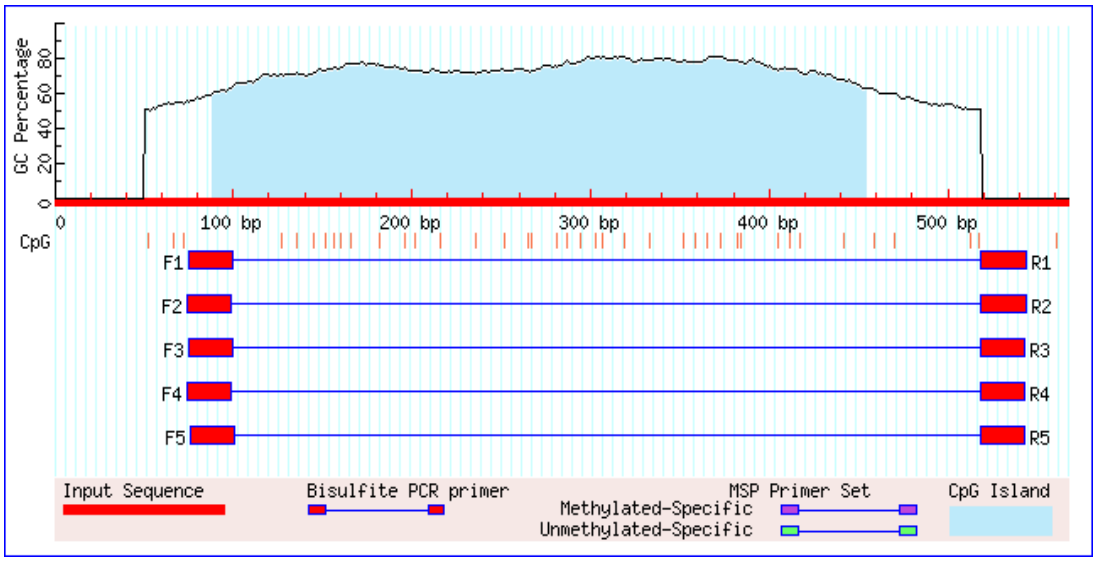

Figure 2.7: Bisulfite Sequencing Primers

Table 2.15: BSS Primer sequences

BSSF	5'- GTTTTTTGGGGGAGAATAAG -3'
BSSMefvR1	5' - TTTCCAAAACCTTCCTTCAAAT -3'

2.5.3.2. BSS PCR CONDITIONS

Since the starting amount of bisulfite treated DNA concentration was low (5ng/µl) two rounds of PCR was needed to amplify the target sequence. First round PCR amplicon was used as a template in the second round PCR. In both PCR reactions, hot start taq was used. The PCR mix and PCR program of first round BSS PCR is given in tables 2.16 and 2.17 whereas the PCR mix and PCR program of the second round BSS PCR is given in tables 2.18 and 2.19.

PCR conditions of the first round of BSS PCR is given in tables 2.16 and 2.17

Table 2.16: PCR mix of first round BSS PCR

Stock Conc.	PCR Ing.	1X	Final Conc.
10X	Hot Star Taq Buffer	5 µL	1X
2mM	dNTP Mix	4 µL	200 µM
10 µM	BSSF	1 µL	0.2 µM
10 µM	BSSMefvR1	1 µL	0.2 µM
-	Bisulfite Treated Genomic Dna	2 µL	-
5u/µL	Hot Star Taq	0.25 µL	
-	dH2O	36.75 µL	
	TOTAL	50 µL	

Table 2.17: PCR program of first round PCR

Initial Denaturation	95°C	15 min	
Denaturation	95°C	30 sec	X35
Annealing	52°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	
Final Hold	4°C	∞	

PCR conditions of the first round of BSS PCR is given in tables 2.18 and 2.19

Table 2.18: PCR mix of second round BSS PCR

Stock Conc.	PCR Ing.	1X	Final Conc.
10X	Hot Star Taq Buffer	5 µL	1X
2mM	dNTP Mix	4 µL	200 µM
10 µM	BSSF	1 µL	0.2 µM
10 µM	BSSMefvR1	1 µL	0.2 µM
-	BSSI Amplicon	2 µL	-
5u/µL	Hot Star Taq	0.25 µL	
-	dH2O	36.75 µL	
	TOTAL	50 µL	

Table 2.19: PCR program of second round PCR

Initial Denaturation	95°C	15min	
Denaturation	95°C	30 sec	X35
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	
Final Hold	4°C	∞	

PCR products were run on 1% agarose gel using 1X TAE buffer.

2.5.3.3. SEQUENCE PCR

In order to obtain the nucleotide sequence of BSS PCR fragments, sequence PCR was done. Sequence PCR protocol is given in table 2.20 and sequence PCR program is given in table 2.21.

Table 2.20: Sequence PCR mix

Stock Conc.	PCR Ing.	1X
5X	Sequence Buffer	2 µL
10 µM	BSSF or BSSMefvR1	1 µL
-	BSS PCR Amplicon	1 µL
5u/µL	Big Dye	2 µL
-	dH2O	4 µL
	TOTAL	10 µL

Table 2.21: Sequence PCR program

Initial Denaturation	95°C	5 min	
Denaturation	95°C	45 sec	X40
Annealing	50°C	45 sec	
Extension	60°C	4 min 30 sec	
Final Hold	4°C	∞	

2.5.3.4. PURIFICATION OF SEQUENCE PCR PRODUCTS

Purification of sequence PCR products were performed according to the protocol given below:

- 10 µl Sequence PCR amplicon was transferred into 1.5ml microcentrifuge tube.
- 2 µl of 3mM NaAC was added to each tube.
- 50 µl of 95% ethanol was added into the tubes, mixed, and remain on ice for 30 minutes.
- Samples are centrifuged 30 min at 14000rpm and supernatant was removed.
- 200 µl of 70% ethanol was added into each tube and vortexed.
- Samples are centrifuged 30 min at 14000rpm and supernatant was removed.
- Tubes are incubated at 95ºC for 5 minutes to allow complete evaporation of remaining ethanol as the covers of the tubes are open.
- 20 µlof formamide was added into the tubes and tubes are vortexed for at least one minute.
- Tubes are centrifuged briefly to collect the sample at the bottom of the tube.

- Samples are incubated at 95ºC for 5 minutes to denaturate the secondary structures of amplicons.
- Samples are immediately put on ice for 5 minutes and kept at 4ºC until loading to capillary gel of sequencer.

2.5.3.5. SEQUENCE DATA ANALYSIS

After the sequencing results are obtained, they were translated using chromas software. Triple alignment was done for methylated bisulfite treated DNA nucleotide sequence , unmethylated bisulfite treated DNA nucleotide sequence, and each sample sequence obtained from sequencing results to see whether the sample's nucleotide sequence matches with methylated one or unmethylated one. For alignment, clustalw software was used. In addition to alignment, chromotogram peak hights of cytosines and/ or thymidines at CpG positions are measured by using an online software called PixelRuler. A table was composed from this peak hight data for each of the 18 DNAs at more than 25 CpG dinucleotide sites. Cytosine peak hights (x) and thymidine peak hights were used to calculate relative cytosine (x/(x+y)) and relative thymidine (y/(x+y)) peak hights. For each DNA, for more than 25 CpG sites, methylation frequency charts were obtained. In addition, methylation differences were compared between wildtype samples and E148Q ones. Methylation results are also compared by MEFV expression levels obtained from another study of human genetics group.

3. RESULTS

3.1. CpG ISLAND ANALYSIS RESULTS

MEFV gene has a GC content of 51% at the genomic DNA level whereas this value is 59% at cDNA level. The second exon contain relatively more guanine and cytosine nucleotides and has a GC content of 66%. Since CpG islands are frequently found at GC-rich parts of the genome, MEFV genomic sequence was analyzed in terms of CpG islans (CpG Island Searcher software, USC). A 998 bp CpG island with a GC content of 61.2 and observed/expected CpG density of 0.653 was found spanning the entire second exon of MEFV gene (Figure 2.1). Within this CpG island, 568 bp region containin 41 CpG sites within its sequence was selected for methylation analysis. CpG sites are given in appendix C.

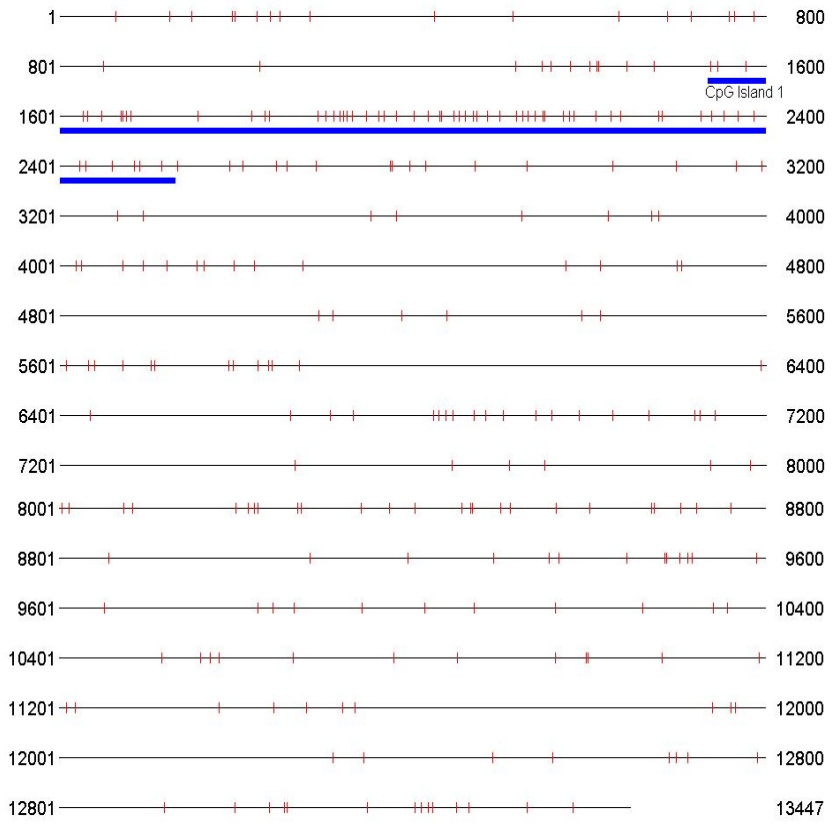

Figure 3.1: CpG island on the MEFV genomic DNA sequence

3.2. E148Q GENOTYPING RESULTS

For E148Q genotyping, 244bp DNA which include the E148Q site was amplified by PCR. PCR product was run on agarose gel and the desired DNA fragment of 244bp was detected. In the figure below, PCR amplicon is shown in figure 3.2.

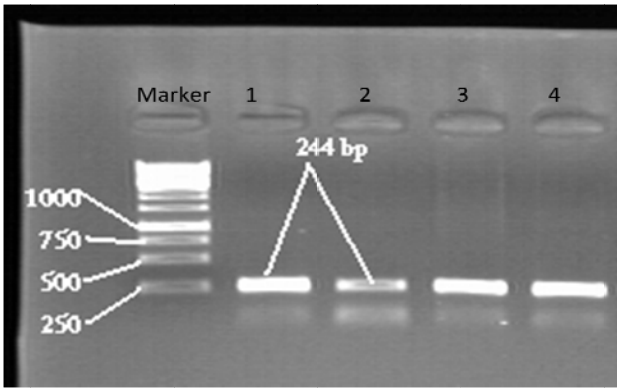

Figure 3.2: PCR-amplified 244bp DNA fragment including E148Q site.

To discriminate between E148Q alleles, AvaI digestion was performed PCR amplified DNA fragments. After AvaI digestion of 244bp DNA fragment, the agarose gel results are obtained and given in figure 3.3.

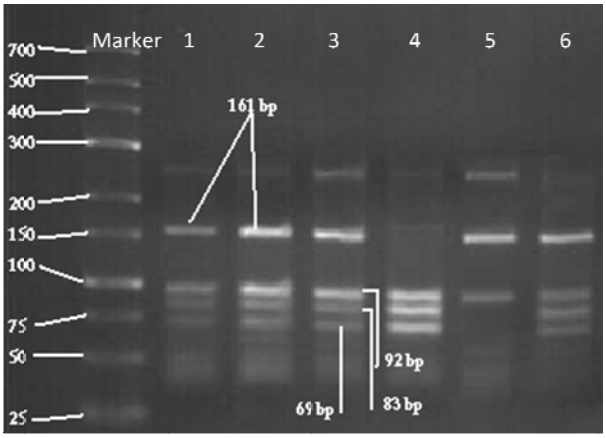

Figure 3.3: AvaI restriction analysis of 6 samples. Lanes 1, 2, 3, and 6 indicate E148Q heterozygote genotype, lane 4 is wildtype whereas lane 5 is homozygote mutant.

According to genotyping results, 15 wildtype and 3 E148Q hetrozygotes were selected for DNA methylation analysis. Wildtype DNAs were named as 153, 154,

155, 156, 157, 158,159, 160, 161, 162, 163,164, 165, 166, and 167 whereas E148Q heterozygote samples were named as 149, 152 and 168.

3.3. METHYLATION SPECIFIC PCR RESULTS

Before setting MSP experiments, methylated and unmethylated MSP positive control fragments were prepared. The unmethylated positive control was PCR amplified from genomic DNA, and observed on the agarose gel which is shown in figure 3.4.

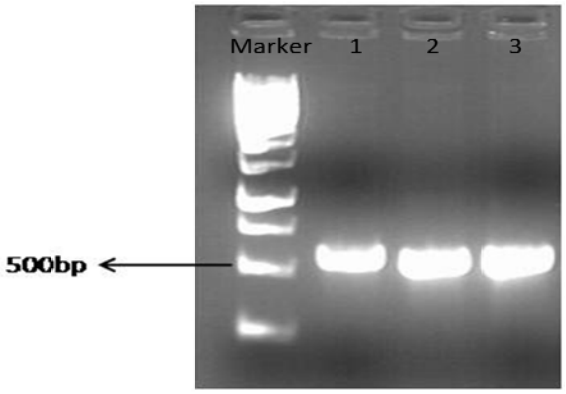

Figure 3.4: PCR amplified control DNA for MSP. Lane 1, 2, and 3 are the same samples of amplified MSP control fragment.

The PCR product obtaned by PCR was purified to a total volume of 100µl, and 50µl of the product was kept at -20ºC to be used as unmethylated conrtol fragments in further MSP experiments. The remaining 50µl PCR product was methylated in vitro by SssI methylase. Quality control of DNA methylation made by BstUI restriction enzyme digestion gived the expected results which are shown in figure 3.5.

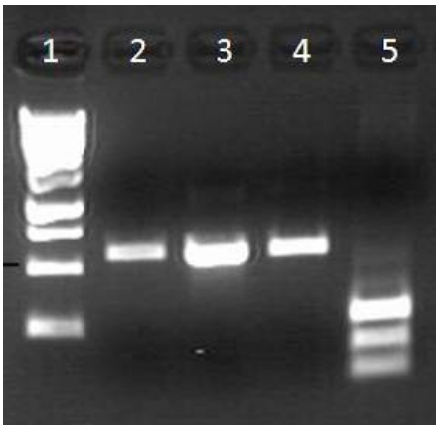

Figure 3.5: BstUI digestion of methylated and unmethylated MSP control DNA fragment. Lane 1 indicate methylated undigested MSP control fragment, lane 2 is unmethylated undigested MSP control fragment whereas lane 3 shows digested methylated MSP control fragment, and lane 4 is unmethylated digested MSP control fragment.

After preparation of methylated and unmethylated controls, MSP was optimized to discriminate two types of control DNAs by using M and U primers. The results of MSP done with control fragments gived the expected pattern which is shown in figure 3.6.

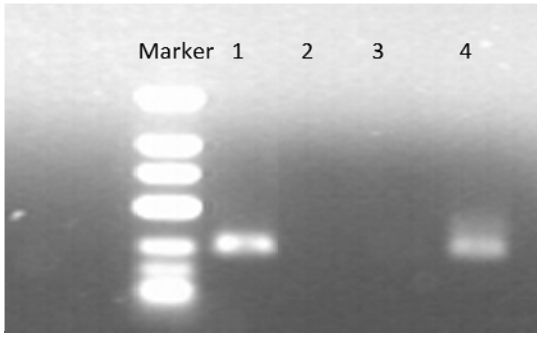

Figure 3.6: MSP results of methylated and unmethylated control DNA fragments. Lane 1 indicate methylated MSP control amplified with M primers and lane 4 indicate unmethylated MSP control fragment amplified with U primers. Lane 2 is the PCR result of methylated MSP control with U primers and lane 3 is the PCR result of unmethylated MSP control with M primers.

After optimization of MSP with methylated and unmethylated control DNA fragments, 18 DNA samples were analyzed in terms of DNA methylation at the second exon of MEFV gene with the same PCR protocol. Methylation Specific PCR results are shown in figure 3.7.

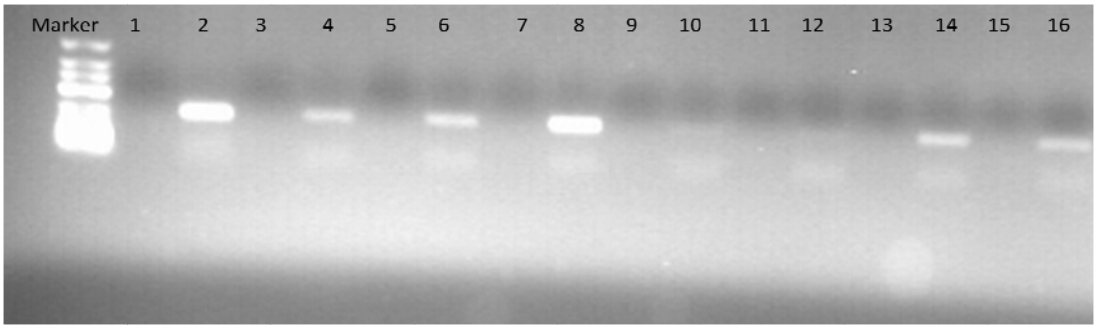

Figure 3.7: Methylation Specific PCR results 8 DNA samples. Lane 1 and 2 are sample 149, lane 3 and 4 are sample 152, lane 5 and 6 are sample 153, lane 7 and 8 are sample 154, lane 9 and 10 are sample 155, lane 11 and 12 are sample 156, lane 13 and 14 are sample 157, and lane 15 and 16 are sample 158.For each sample, first lane is amplified bu U primers whereas second one is amplified by M primers.

3.4. BISULFITE SEQUENCING RESULTS

BSS PCR was performed in addition to MSP to confirm the results of MSP. Besides, BSS PCR fragments are further analyzed by DNA sequncing to quantify CpG methylation. Bisulfite sequencing PCR results of 18 bisulfite treated DNA samples are shown in figure 3.8.

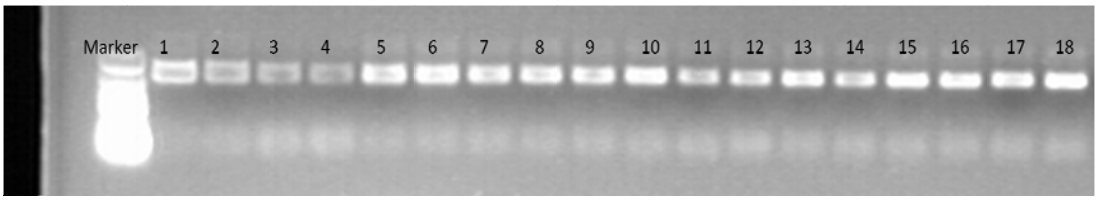

Figure 3.8: Bisulfite sequencing PCR-amplified DNA fragments for 18 DNA samples. From lane 1, the samples are 149, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, and 168.

After DNA sequencing of each BSS PCR fragment, the results obtained by sequencing was aligned with methylated and unmethylated – bisulfite treated DNA samples. Sequence alignment results obtained from each of the 18 DNA sample were given in the appendix D.

DNA sequencing of BSS amplicons were achieved succesfully with a very low level of background peaks. For each of the 18 DNA samples, CpG methylation map was established for each of the 41 CpG sites within the analyzed sequence (Appendix E). CpG sites are seen in the forward primer-red sequence as overlapping cytosine (blue)

and tymidine (red) peaks. In the case of reverse primer-red sequences, CpG sites are seen as overlapping guanine (black) and adenine (green) peaks. Overlapping peaks indicate partial DNA methylation whereas single cytosine (in forward primer-red sequence, figure 3.9) or single guanine (in reverse primer-red sequence, figure 3.10) peaks indicate a complete (100%) methylation at that CpG site.

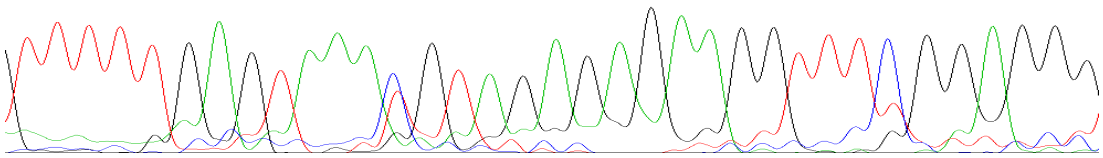

Figure 3.9: Forward primer-red BSS sequence

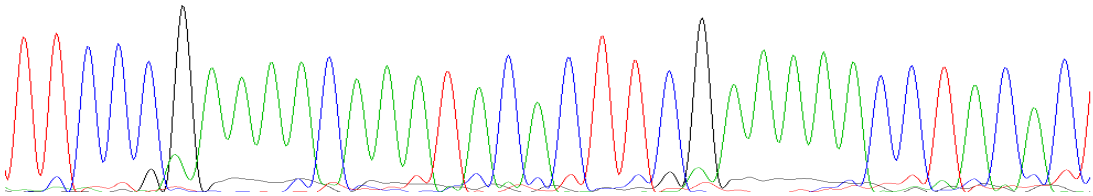

Figure 3.10: Reverse primer-red BSS sequence

3.5. DNA METHYLATION AND MEFV EXPRESSION ANALYSIS

Obtained DNA methylation patterns of the second exon of MEFV gene was compared to MEFV gene expression levels of individuals. Two subgroups which are E148Q hetrozygotes and wildtypes were also compared to eachother in terms of second exon methylation (Figure 3.11), and CpG methylation at E148Q site (Figure 3.12).

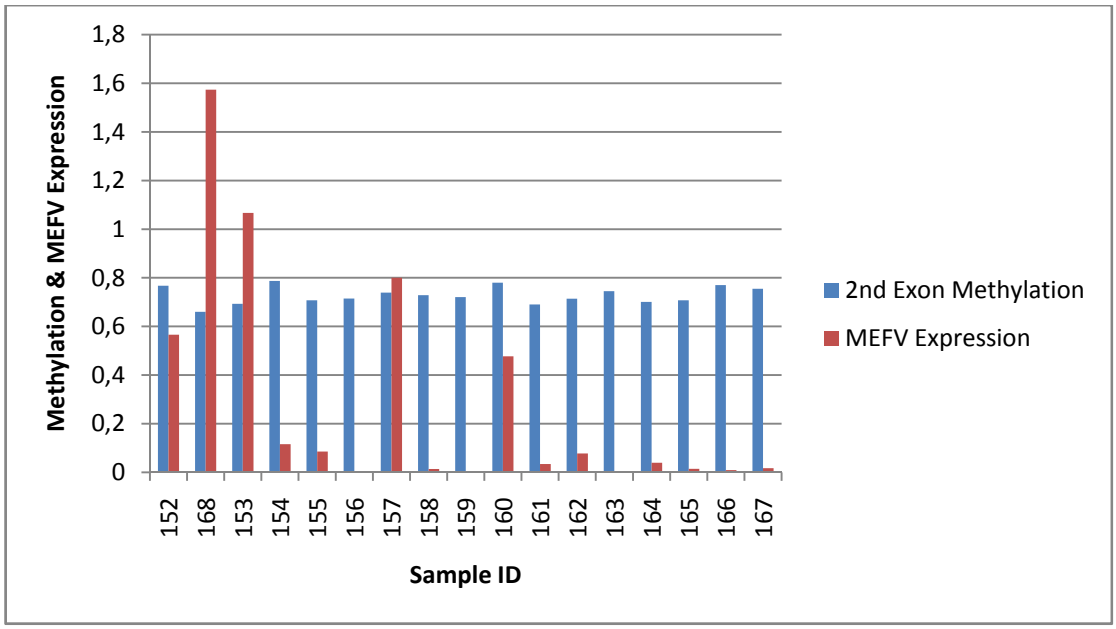

Figure 3.11: Global methylation at the second exon of MEFV gene compared to MEFV gene expression levels.

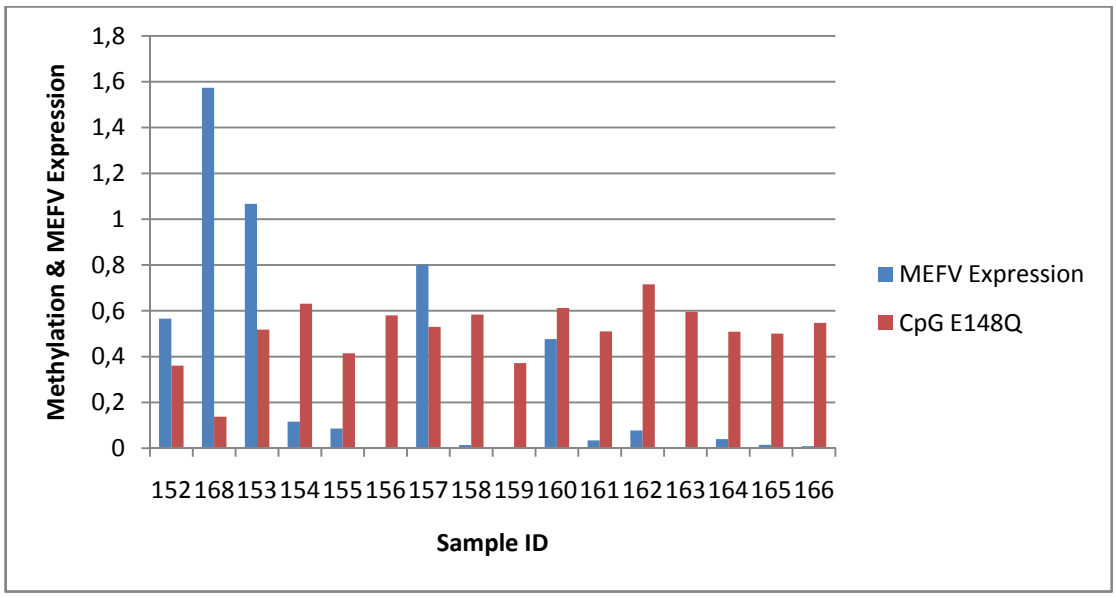

Figure 3.12: CpG methylation at E148Q site compared to MEFV gene expression.

For 18 samples analyzed, a CpG pattern throughout the second exon of MEFV gene was obtained by using the average CpG methylation of each CpG site in all of the samples (wildtypes together with heterozygotes). The results are given in the figure 3.13.

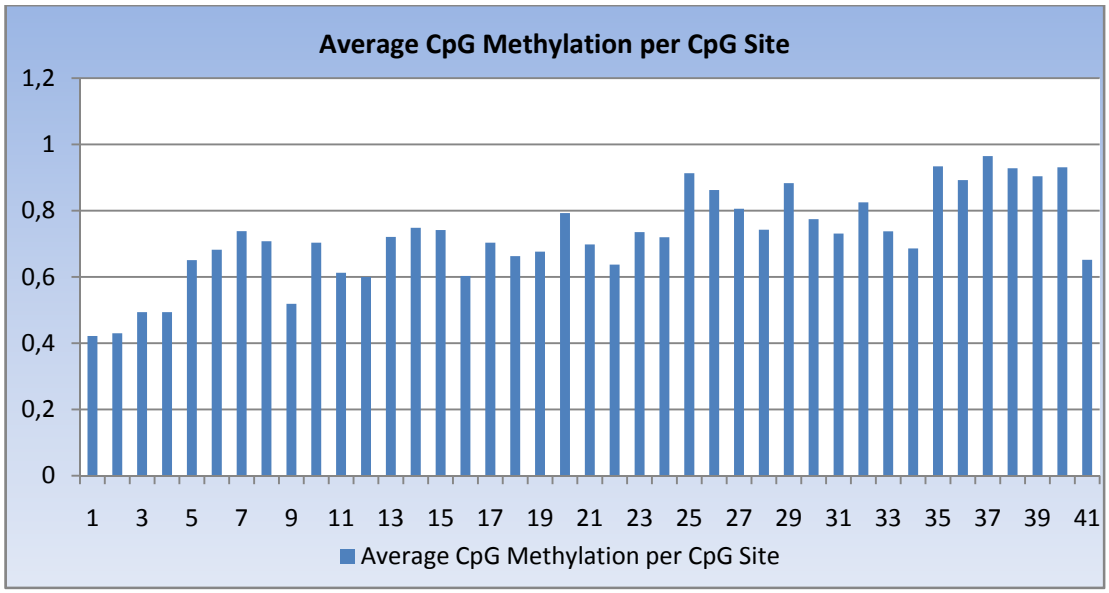

Figure 3.13: Average CpG methylation for each CpG site throughout the second exon of MEFV gene. Methylation level of each CpG is the average of wildypes and heterozygotes together.

Determination of global methylation pattern of the second exon of MEFV gene revealed that there is a difference between the first half of the exon and the second half of the exon. The comparison of average methylation of the first half of the second exon and average methylation of the second half of the second exon were compared both in wildtype and E148Q heterozygote samples. The results are shown in figure 3.14.

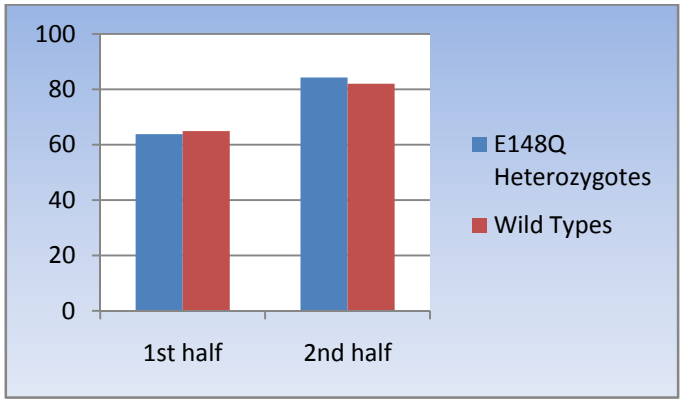

Figure 3.14: Comparison of average DNA methylation between the first half and the second half of the second exon of MEFV gene.

To compare the global methylation of second exon between wildtype and E148Q heterozygotes, the average DNA methylation of each CpG at the second exon of wildtypes are compared to heterozygote ones. The data obtained from this comparison is given in figure 3.15.

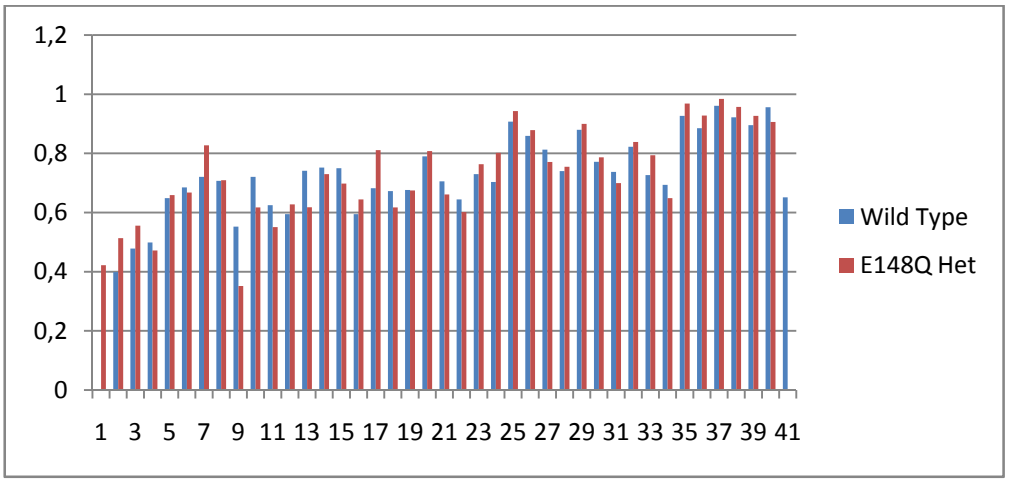

Figure 3.15: DNA Methylation comparison between wildtype and E148Q heterozygotes for each CpG site.

In order to check whether second exon methylation levels have a relationship with MEFV expression, wildtype and heterozygote samples are compared in terms of average second exon methylation and average MEFV gene expression. The results are given in figure 3.16.

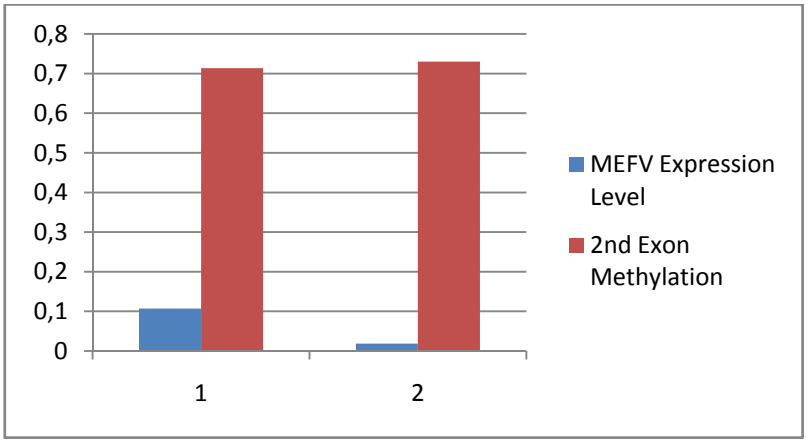

Figure 3.16: Comparison of average DNA methylation of second exon of MEFV gene and average MEFV expression between wildtype and E148Q heterozygote samples.

To determine whether CpG methylation level of E148Q site have a relationship with MEFV expression, wildtype and heterozygote samples are compared in terms of average CpG methylation of E148Q site and average MEFV gene expression. The results are given in figure 3.17.

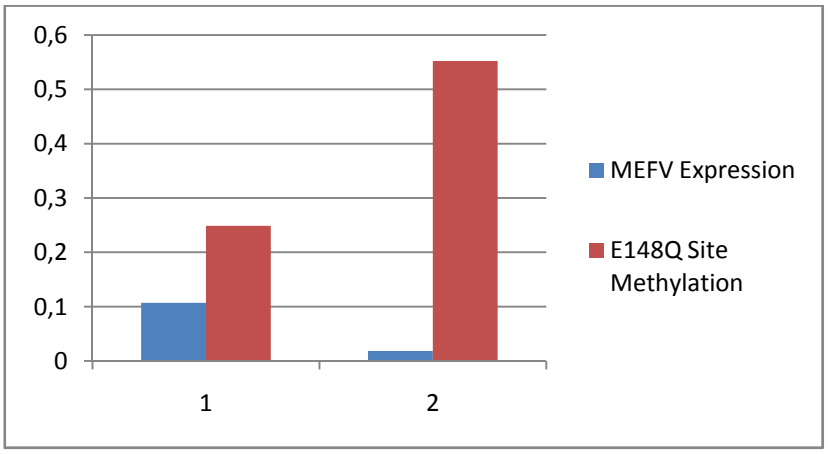

Figure 3.17: Comparison of average DNA methylation of E148Q site and average MEFV expression between wildtype and E148Q heterozygote samples.

In addition to comparison between E148Q heterozygotes and wildtype samples, 9 FMF samples were compared with 15 healthy controls which do not have FMF disease. MEFV expression levels of these samples were also analyzed in connection with their second exon methylation (Figure 3.18) and methylation of CpGs at positions 9 10 and 11 (which can be seen in appendix C) that is given in figure 3.19.

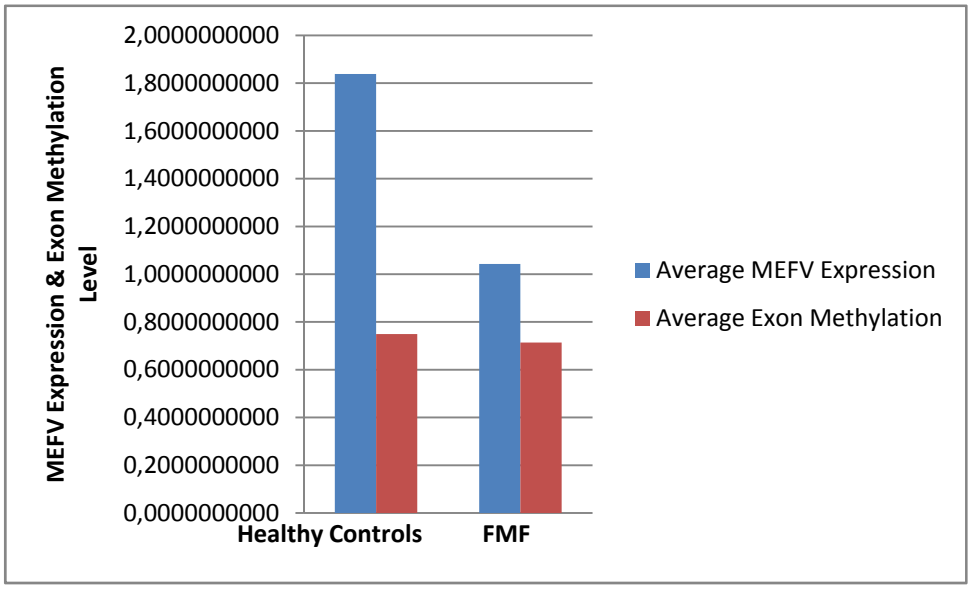

Figure 3.18: Average MEFV Expression vs Average Exon Methylation Comparison Between FMF Samples (N=9) and Healthy Controls (N=15)

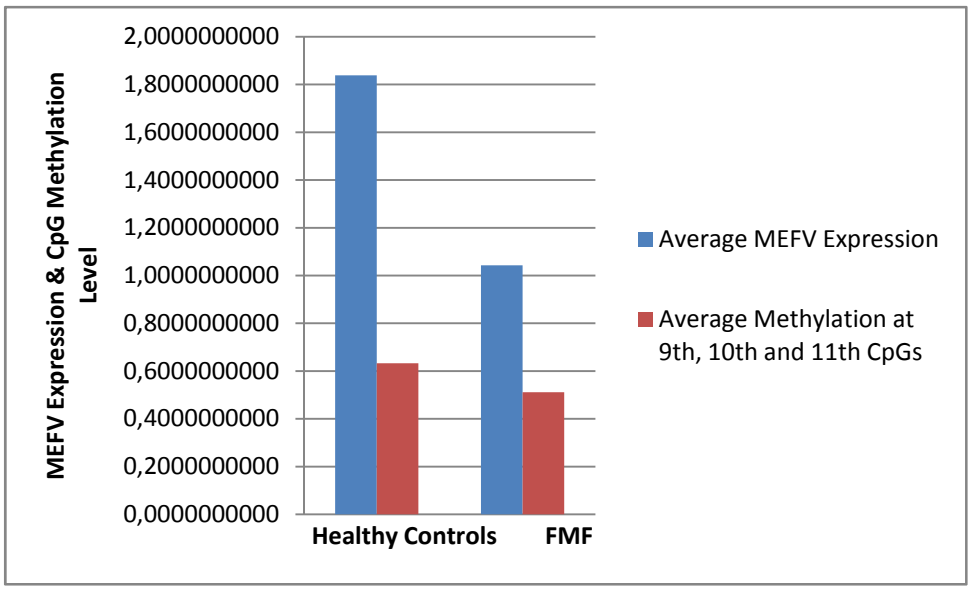

Figure 3.19: Average MEFV Expression vs Average CpG Methylation (at 9th, 10th and 11th Position) Comparison Between FMF Samples (N=9) and Healthy Controls (N=15)

4. DISCUSSION

In this study, DNA methylation analysis was established by Methylation Specific PCR and direct bisulfite sequencing techniques. The target region of methylation analysis was the CpG island spanning the second exon of MEFV gene.

To determine the methylation pattern of the second exon of MEFV gene, bisulfite treatment was performed to modify unmethylated cytosines into thymidines. The efficiency of bisulfite treatment is important for complete conversion of unmethylated cytosines into thymidines. Otherwise, some unmethylated cytosines remain the same and may give false positives. The control of bisulfite treated – MSP amplified DNA fragment showed a complete conversion for 6 unmethylated cytosine residues within the amplified sequence. On the other hand, according to bisulfite sequencing results, at non-CpG sites all of the cytosine residues are completely converted to thymidine residues which indicate a high yield for bisulfite conversion in this study.

Positive controls of Methylation Specific PCR was also gived the expected amplification pattern with MSP. M primers amplified only methylated, bisulfite treated DNA but not unmethylated, bisulfite treated DNA; whereas U primers amplified only unmethylated, bisulfite treated DNA but not methylated, bisulfite treated DNA. Establishment of MSP with these positive control primers is essential in terms of the reliability of methylation analysis of DNA samples.

MSP analysis of 18 DNA samples showed that in all of the samples, M primers amplified the target sequence whereas U primers not with a 35-cycle PCR reaction. This preliminary data showed that the second exon of MEFV gene is highly methylated.

During the establishment of bisulfite sequencing technique, it was seen that the initial template DNA amount plays a key role on bisulfite sequencing PCR. Thus, first

round PCR did not amplified the target bisulfite sequencing fragment, a second round of PCR was needed using the first PCR amplicon as template DNA.

For both MSP and BSS PCR amplifications, it was seen that the hot start PCR technique is essential for amplification. Hot start PCR technique with Taq DNA Polymerase (fermentas) and classic PCR technique with HotStart Taq (Takara) were both worked very well, whereas no amplification was obtained in classical PCR technique with Taq DNA Polymerase (Fermentas).

Bisulfite sequencing was done for each sample with a forward and with a reverse primer to set internal control of methylation quantification for BSS technique. In all of the DNA samples, reverse primers worked better than forward primers, which is a common case in BSS. For forward primers, 10 out of 18 sequencing results could't be obtained. For the 8 samples which have two sequencing results with forward and with reverse primers, the methylation frequency of certain CpGs at the overlapping sites were compared in order to see whether there is a difference between two BSS results of forward and reverse primers for the same DNA fragment. The results showed that there is perfect match between the methylation pattern determined with forward primer and with reverse primers at he indicated CpG sites, which show the reliability of the BSS methylation quantification.

According to the CpG methylation analysis with BSS technique, approximately 35 CpG sites were characterized in terms of DNA methylation for each of the 18 DNA samples. 15 of the samples were wildtype whereas 3 of the samples were E148Q heterozygotes. E148Q mutation eliminates a CpG site and thus expected to decrease DNA methylation at mutation site.

The results of alignments of bisulfite sequencing data showed that when methylated and unmethylated control sequences are compared, 95% of the DNA samples were directly matched with the methylated DNA pattern which confirmed MSP results.

A common methylation pattern was observed througout the CpG island nearly all of the 18 samples, where the methylation was relatively higher at 7th, 14th, 20th, 25th, 29th, and 35th CpG positions compared to the rest of the sequence. The 9th CpG is the E148Q site. A slight decrease was observed at the 9th CpG in heterozygotes. Average CpG methylation at the 9th CpG is 0.35 in E148Q heterozygotes whereas

this value is 0.55 in wildtypes. On the other hand, average CpG methylation of second exon was found to be the same between wildtype and heterozygote samples (73%) which indicate E148Q mutation does not not have any effect on global DNA methylation of the second exon.

Another important data obtained from BSS methylation quantification is that, independent of the genotype, the second half of the exon was slightly more methylated than the first half. Since the first half, especially the first 3 CpGs at the analyzed site are more close to the 5' UTR of the MEFV gene, methylation amount of that CpGs may be lower due to positional effect relative to the promoter site.

DNA methylation levels were also compared with the MEFV expression data. No relationship was found between the global methylation levels of the second exon of MEFV gene and MEFV expression between wildtype and E148Q heterozygote subgroups. However, when witldtype and E148Q hetrozygotes were compared, methylation amount of 9th CpG (E148Q site) was found to be strongly associated with MEFV expression. CpG methylation at the site of E148Q mutation was found to be reversely proportional with MEFV gene expression. Additionally, in nearly all of the samples, average DNA methylation was higher than 50% whereas this value was significantly lower in the first 3 CpGs.

Moreover, DNA methylation patterns of the second exon of MEFV gene was compared between FMF samples and healthy controls. The exon-wide methylation pattern (for the second exon of MEFV gene) wasn't found to be significantly different between these subgroups. However, CpGs at positions 9, 10, and 11 were found to have 10 to 15 percent lower methylation level when compared to healthy controls. CpG methylation versus MEFV experiments between these two subgroups did not give any correlation with MEFV expression and DNA methylation pattern of the second exon of MEFV gene.

5. CONCLUSION AND FURTHER COMMENTS

MEFV was identified as the candidate gene of FMF disease. There are 5 common mutations within MEFV gene which were found to be associated with disease pathology. One of these mutations, E148Q was shown to increase MEFV expression, which causes a milder phenoype and also suggested to be preventive against amyloidosis. These data support that this polymorphism may be functional.

When MEFV gene is analyzed by bioinformatic tools, a CpG island spanning the second exon and containing the E148Q site was found. By using DNA methylation analysis techniques like methylation specific PCR and Bisulfite Sequencing, methylation pattern of this CpG island was established during this study. It was found that CpG methylation is lower at the first parts of the CpG island, which is closer to the promoter site. The second half of the CpG island was heavily methylated compared to first half.

Since E148Q mutation eliminates a CpG site within this CpG island, DNA methis methylation levels of wildtype and E148Q heterozygote samples were compared to see if there is a difference between methylation levels between thses subgroups. In terms of global CpG island methylation, no difference was observed, but at E148Q site, a slight decrease in DNA methylation was observed in E148Q heterozygotes comnpared to wildtype samples. The methylation data was also compared with the MEFV gene expression levels obtained by another study of Human Genetics Group. According to this analysis, MEFV gene expression was shown to reversely proportional with E148Q site methylation.

In this study, 15 wildtype and 3 E148Q heterozygote samples were analyzed in terms of methylation of second exon of MEFV gene. As a further aspect, the number of samples can be increased to obtain more reliable results. Analyzing DNA methylation pattern of E148Q homozygote mutant samples can give additional information.

In addition, since MEFV levels were shown to be decreased in FMF patients carrying some MEFV mutations, DNA methylation pattern were compared between FMF samples and healthy controls to check whether an epigenetic regulation mechanism lies under the disease pathology. According to the results, no significant difference was found in the methylation pattern of the second exon of MEFV gene between FMF samples and healthy controls. At CpGs 9, 10 and 11 there will be a 10-15% decrease in CpG methylation and this can be further studied in terms of MEFV gene expression regulation.

Finally, these in vivo methylation studies can be confirmed by in vitro cell culture studies which can be also useful for further methylation & gene expression experimets.

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APPENDIX A

EQUIPMENT

Balances	Precisa 620C SCS
	Precisa BJ 610C
Centrifuges	Sigma 1-13 B. Braun International
	Allegra 25R Centrifuge Beckman Coulter
Electrophoresis equipments	E – C mini cell primo EC320
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows
Pipettes	Gilson Pipetman 20 µL 200 µl, 1000 µl
	Thermo Finnpipette 10 µL,
pH meter	Mettler Toledo MP220
Spectrophotometer	PerkinElmer Lambda25 UV/VIS Spectrometer
Thermo cycler	Applied Biosystems GeneAmp PCR
	System 2700
	Corbett PalmCycler
	Techne FTGENE 5D
Transilluminator	Biorad UV Transilluminator 2000
Vortex	Herdolph Reax top

APPENDIX B

B.1. CHEMICALS

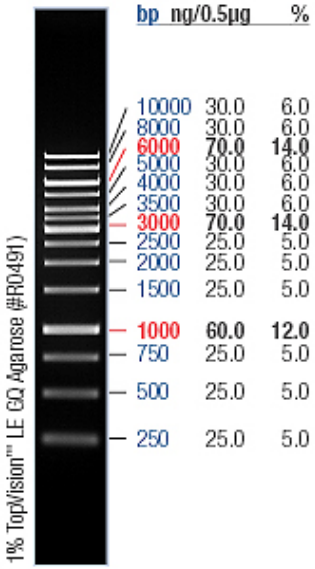
Agarose	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
Ethanol	Riedel-de Haën
EtBr	Amresco
$MgCl_2$	Fermentas
	Takara
Primers	IDT DNA
Tris Base	Amresco
10X PCR Buffer	Fermentas
	Takara

B.2. ENZYMES

Ava*I*	Fermantas
Taq DNA Polymerase	Fermentas
	Takara

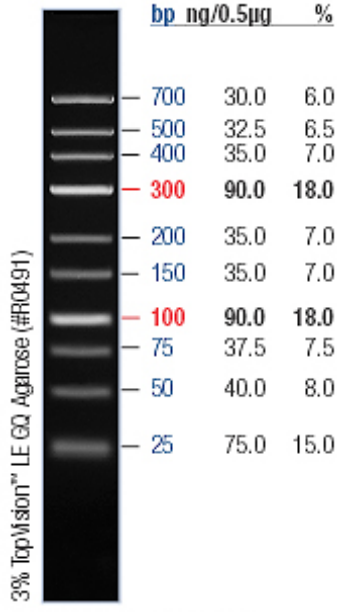
B.3. MARKERS

Gene Ruler™ 1 kb DNA Ladder Fermentas



0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

Gene Ruler™ DNA Ladder Low Range Fermentas



0.5µg/lane, 8cm length gel,
1X TBE, 5V/cm, 1h

B.4. BUFFERS

TE Buffer

Tris base	10 mM
EDTA	1 mM

Add ddH_2O to 1 liter and adjust the pH to 8.0

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris base	108 g
Boric Acid	55 g
EDTA	40 ml (0.5 M, pH 8.0)

Add ddH_2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose	0.5 g
TBE buffer (1X)	50 mL

Add 1.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Midi Agarose Gel (1%)

Agarose	1.5 g
TBE buffer (1X)	150 mL

Add 4.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

B.5. USED KITS

DNA Isolation	8 Lx Magtration® Genomic DNA kit
Bsiulfite Treatment	Qiagen EpiTect Bisulfite Kit

APPENDIX C

CpG SITES ON BSS-ANALYZED SEQUENCE

```
Methylated          GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
Unmethylated        GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60
                    ************************************************ ******** *

Methylated          AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
Unmethylated        AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120
                    ******* ****** *** *** ***** *************** ************* *

Methylated          AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
Unmethylated        AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                    **** ************* ****************** *************** *****

Methylated          GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
Unmethylated        GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                    ******* * ************* ***** ****** ******** *** **********

Methylated          TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
Unmethylated        TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                    * ************* ***************** ****** ***** ******* ****

Methylated          GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
Unmethylated        GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                    **** * ******************** ****** **** ********************

Methylated          TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
Unmethylated        TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                    **** ***************** ********** ****************************

Methylated          TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
Unmethylated        TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                    *************** *** ****************************************

Methylated          TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
Unmethylated        TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                    *** *********** ************* *************** ***************

Methylated          TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
Unmethylated        TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
                    *** ************************
```

APPENDIX D

BSS ALIGNMENT FILES OF 18 SAMPLES

(M indicate methylated bisulfite treated DNA, U indicate unmethylated, bisulfite treated DNA, and S indicate Sample)

Sample 149

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 1S0
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 1S0


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------GGAGG 5
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                                                                     *****

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT S40
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 65
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT S40
                ******* * ************* ***** ****** ******** *** **********

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 1S5
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ***************** ****** ***** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 185
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 4S0
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT S45
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 4S0
                **** *************** ********** **************************

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 305
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGG-------------------- 344
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ************* ********

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 152

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60

M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120

M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ----------GGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 50
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                         ******** ****************** *************** *****

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 2S0
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 110
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 2S0
                ******* * ************* ***** ****** ******** *** **********

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 170
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAAGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 230
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ***** ************** ****** **** ********************

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT S20
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 290
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT S20
                **** *************** ********** ***************************

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG S80
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 350
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG S80
                *************** *** ****************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 5S0
S               TTGCGAATTTGGATTCGGTAATAGAATTTCGGG-------------------------- 383
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 5S0
                *** *********** ************* ***

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 153

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG S0
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG S0


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               -----------------------------------GGTTTTGTCGGGCGGGAGAAGTT 23
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                   ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 83
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 3S0
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGGTGTTTCGGGGTAGAAGGAGTGTAGGT 143
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 3S0
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTTGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 203
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 2S3
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATA----------------------------------- 28S
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** *******


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 5S8
S               ----------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 5S8
```

Sample 154

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 1S0
S               ------------------CGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 42
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 1S0
                                    ******************* ************** *****


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 102
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                ******* * ************* ***** ****** ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 162
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 222
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 2S2
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** **************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 4S0
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 342
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 4S0
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAA---------------------------------------------------- 349
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** ***


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 56S
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 56S
```

Sample 155

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------GAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 47
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60
                                  ********************************** ******** *

M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGG-AGTTGTTAGTTTGCGGTGTAGTTAGTTC 119
S               AGGGGAATGGTTTTTGGTCGTACGGGGGCGGGAGTTGTTAGTTTGCGGTGTAGTTAGTTT S 7
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGG-AGTTGTTAGTTTGTGGTGTAGTTAGTTT 119
                ******* ****** *** *** ***** ** ************* *************

M               GAGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAG 179
S               GAGGTCGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTATCGGAG 167
U               GAGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAG 179
                ***** ************* ****************** ************* * ****

M               GGTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGT 239
S               GGTTTGGATGCGTAGGGTAAGTTTTGGATTCGGAGTTCGGTTTTGTTGGGCGGGAGAAGT 227
U               GGTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGT 239
                ******** * ************* ***** ****** ******** *** *********

M               TTCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTT 299
S               TTCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTT 287
U               TTTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTT 299
                ** ************* ****************** ****** ***** ******* ***

M               AGTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGG 359
S               AGTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGG 347
U               AGTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGG 359
                ***** * ******************** ****** **** ******************

M               TTTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATT 419
S               TTTTTCGAAGTGTATTTGTTTTTGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATT 407
U               TTTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATT 419
                ***** ***************** ********** *************************

M               TTTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATA 479
S               TTTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATA 467
U               TTTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATA 479
                **************** *** ************************************

M               GTTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTA 539
S               GTTGCGAATTTGGATTCGGTAATAGAATTTCGGG------------------------- 501
U               GTTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTA 539
                **** *********** ************* ***

M               TTTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------- 
U               TTTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 156

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG S 0
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG S 0


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               ----------------------------------------------GGGAGAAGTT 10
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                               **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 70
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ***************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 130
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************* ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTCTTCTTT 190
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ******************* ** ***


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 250
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ****************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGA------------------------------------------- 263
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 157

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               -----------------------------------GGTTTTGTCGGGCGGGAGAAGTT 23
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                   ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 83
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT S 3
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 203
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 263
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTT------------------------------ 292
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** *************


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 158

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60

M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120

M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               -------------------------------------------CGGGCGGGAGAAGTT 15
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                           *** **********

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 75
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGGAGGAGTGTAGGT 135
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ******* ************

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 195
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** **************************

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 255
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTCTG---------------------------- 286
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ************  *

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 159

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ----------------------------GGAGTTGTTAGTTTGCGGTGTAGTTAGTTTG 31
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120
                                            ********************* ************ *


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG S 0
S               AGGTCGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 91
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG S 0
                **** ************* ****************** ************** *****


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTTGGGCGGGAGAAGTT 151
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                ******* * ************* ***** ****** ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 211
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ***************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 271
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************* ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 331
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 391
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTCGGG-------------------------- 424
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ************* ***


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 160

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 1S
S               ----------------------------------------------GTAGTTAGTTCG 12
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 1S
                                                               ********** *


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               AGGTCGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 72
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                **** ************* ****************** *************** *****


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 132
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                ******* * ************* ***** ****** ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 192
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 252
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 4S
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 312
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 4S
                **** *************** ********** **************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 372
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAG---------------------------------- 396
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ********


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 161

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60

M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120

M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               -----------------------------------------------GGGAGAAGTT 10
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                               **********

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 70
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGCGGTAGAAAGAGTGTAGGT 130
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** * ******* **********

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 190
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 250
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAAT-------------------------------- 277
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ***********

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 162

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------TCG 3
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120
                                                                      * *

M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTCGGAGG 63
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                **** ************* ****************** *************** *****

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT S 0
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 123
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT S 0
                ******* * ************* ***** ****** ******** *** **********

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCAGTTGCGTAGAAACGTTA 183
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ***************** ******  **** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAAGAGTGTAGGT S 3
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********* **********

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 303
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 363
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTC----------------------------- 393
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** *************

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 163

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------GAGG 4
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                                                                      ****


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 64
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                ******* * ************* ***** ****** ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TTGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 124
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 184
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 244
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 304
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAAT-------------------------------- 331
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ***********


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 164

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               ----------------------------------------------------GGAGAAGTT 9
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                                    *********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTTAGTTGCGTAGAAACGTTA 69
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ******  **** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAAGAAGAGTGTAGGT 129
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ******  * **********


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 189
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** **************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGA----------------- 231
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** **********************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               ------------------------------------------------------------
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 165

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               ----------------------------------------------GGCGGGAGAAGTT 13
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                               ** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA S 0
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTTAGTTGCGTAGAAACGTTA 73
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA S 0
                * ************* ***************** ******  **** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGGAGGAGTGTAGGT 133
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ******* ************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTTGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 193
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 253
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTCGGG-------------------------- 286
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ************* ***


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 166

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60

M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120

M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180

M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               ------------------------------------------------------------
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240

M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               ----TTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 56
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                    *********** ***************** ****** ***** ******* ****

M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 116
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************* ****** **** ********************

M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTACTATTT 176
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ********************** *****

M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 236
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************

M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTC----------------------------- 266
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** *************

M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

Sample 167

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               ------------------------------------------------------------
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               ----------------------------------------------GGCGGGAGAAGTT 13
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                                                              ** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 73
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ****************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 360
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 133
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT 360
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 193
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 253
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGA---------------------------------- 278
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** *********


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

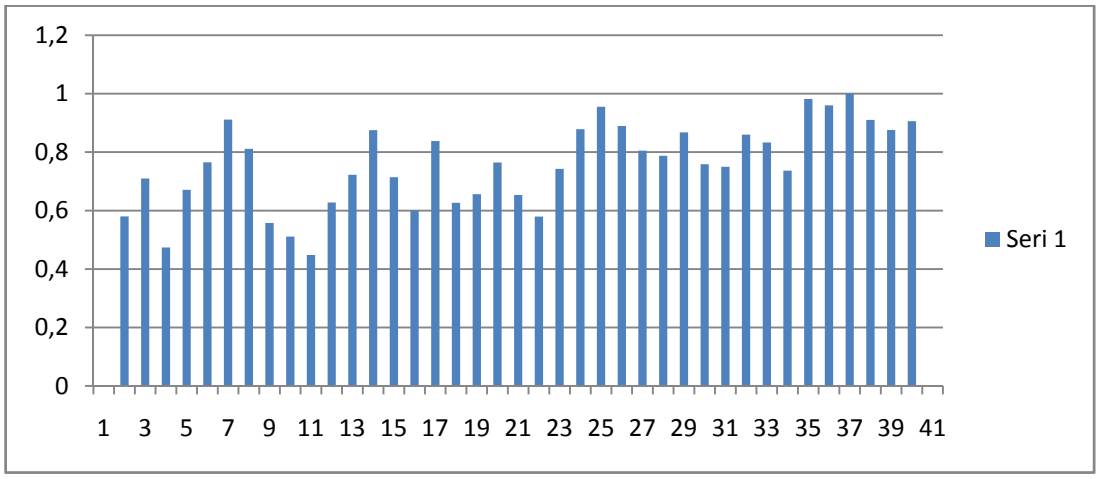
Sample 168

```
M               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTCGAGGGGAACG 60
S               ------------------------------------------------------------
U               GTTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTTAGATTATTTTGAGGGGAATG 60


M               AGGGGAACGGTTTTCGGTCGTACGGGGGCGGAGTTGTTAGTTTGCGGTGTAGTTAGTTCG 120
S               ------------------------------------------------------------
U               AGGGGAATGGTTTTTGGTTGTATGGGGGTGGAGTTGTTAGTTTGTGGTGTAGTTAGTTTG 120


M               AGGTCGGGAGGGGGTTGTCGAGGAAGTTTTTGAGTAAACGTAGAGAGAAGGTTTCGGAGG 180
S               -------------------------------------------AGAAAGGTTTCGGAGG 16
U               AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAGTAAATGTAGAGAGAAGGTTTTGGAGG 180
                                                           ******* *****


M               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTCGGGCGGGAGAAGTT 240
S               GTTTGGACGCGTAGGGTAAGTTTCGGATTCGGAGTTCGGTTTTGTTGGGCGGGAGAAGTT 76
U               GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT 240
                ******* * ************* ***** ****** ******** *** **********


M               TCGGTTTTTGTAGGGCGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 300
S               TCGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTCGAGGTTCGGTTGCGTAGAAACGTTA 1S
U               TTGGTTTTTGTAGGGTGTTAGAGGGGGGTTAGGTTGAGGTTTGGTTGTGTAGAAATGTTA 300
                * ************* ***************** ****** ***** ******* ****


M               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT S 0
S               GTTTCGCGGGGAGGTTGTAGGGGTTGGCGGGGGGCGTTTCGGGGTAGAAGGAGTGTAGGT 196
U               GTTTTGTGGGGAGGTTGTAGGGGTTGGTGGGGGGTGTTTTGGGGTAGAAGGAGTGTAGGT S 0
                **** * ******************** ****** **** ********************


M               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGCGATTTAGAAGTTTTGAGGTTATTATTT 420
S               TTTTCGAAGTGTATTTGTTTTCGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 256
U               TTTTTGAAGTGTATTTGTTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT 420
                **** *************** ********** ***************************


M               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
S               TTATAGGGGAGAAGGCGTTCGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 316
U               TTATAGGGGAGAAGGTGTTTGTAAATTTAGAAATTTTTTTGATTTTAGAGGAAAAGATAG 480
                *************** *** ************************************


M               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTAAGGTTTATTTCGGATGGAGGGGTAT 540
S               TTGCGAATTTGGATTCGGTAATAGAATTTCGGGTA------------------------- 351
U               TTGTGAATTTGGATTTGGTAATAGAATTTTGGGTAAGGTTTATTTTGGATGGAGGGGTAT 540
                *** *********** ************* *****


M               TTGCGGATTTGAAGGAAGGTTTTGGAAA 568
S               ---------------------------
U               TTGTGGATTTGAAGGAAGGTTTTGGAAA 568
```

APPENDIX E

CpG METHYLATION MAPS OF 18 SAMPLES

149



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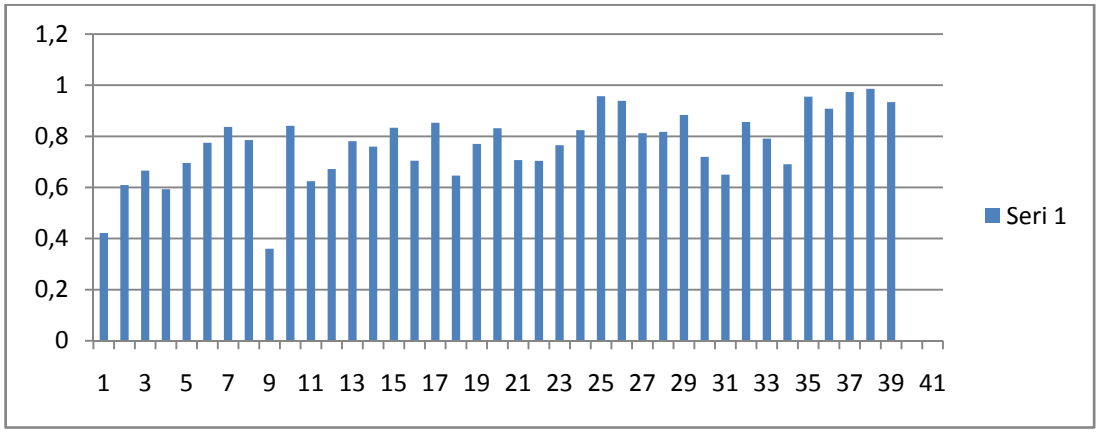

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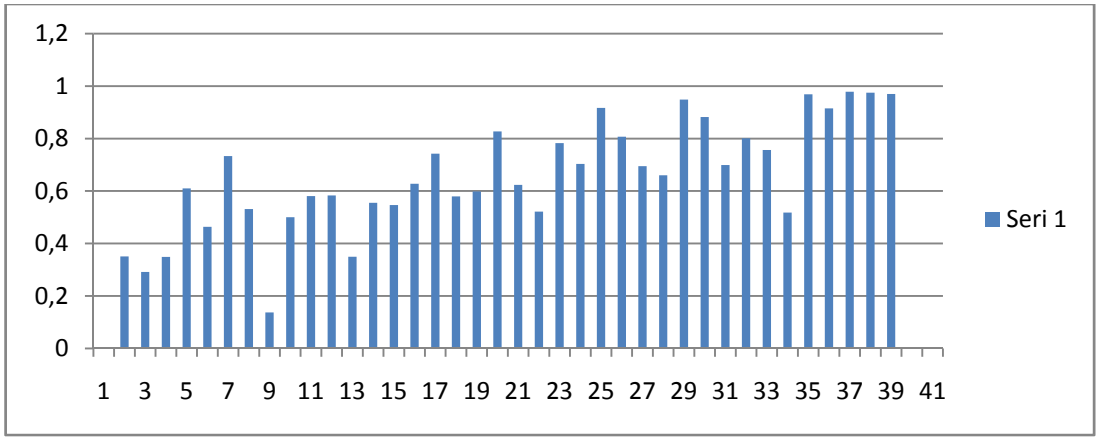
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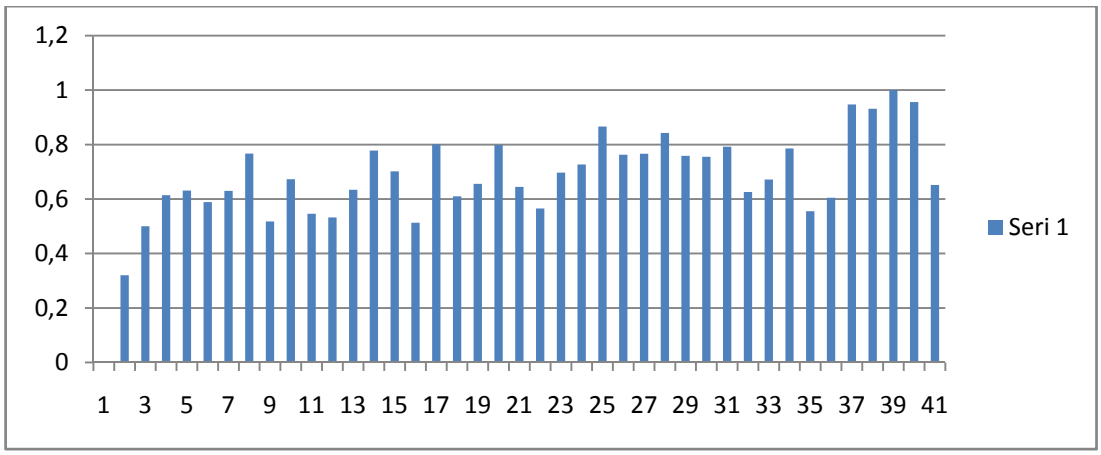
Seri 1

153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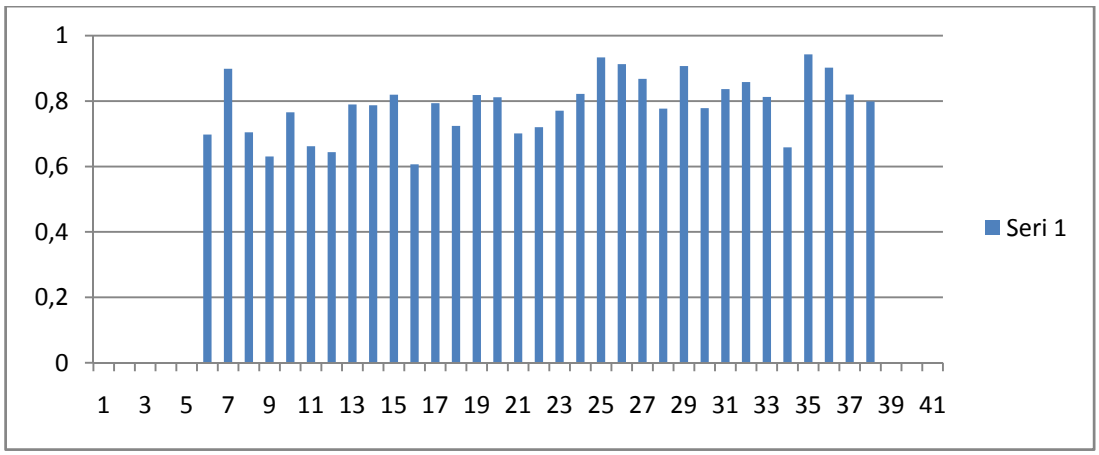
Seri 1

154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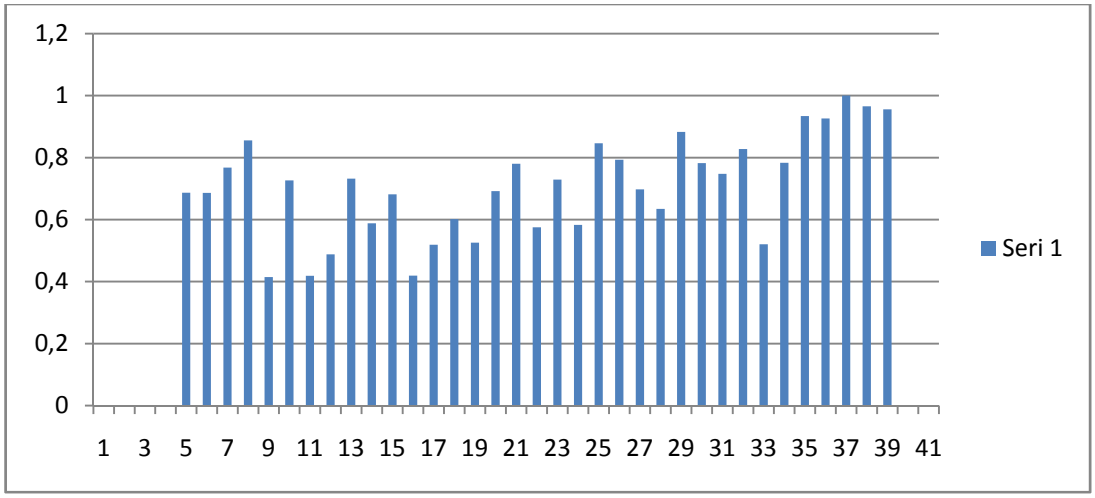
155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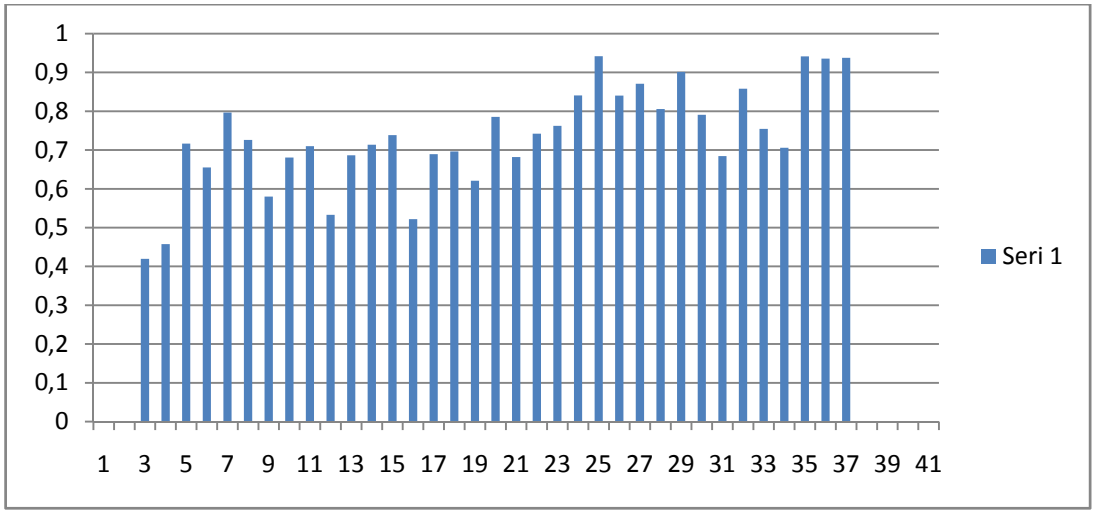
156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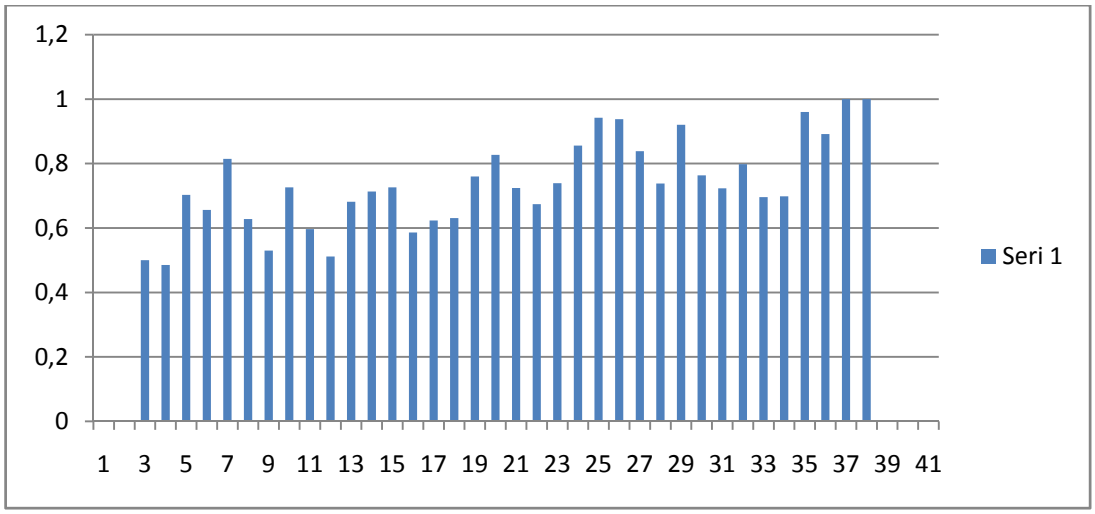
Seri 1

157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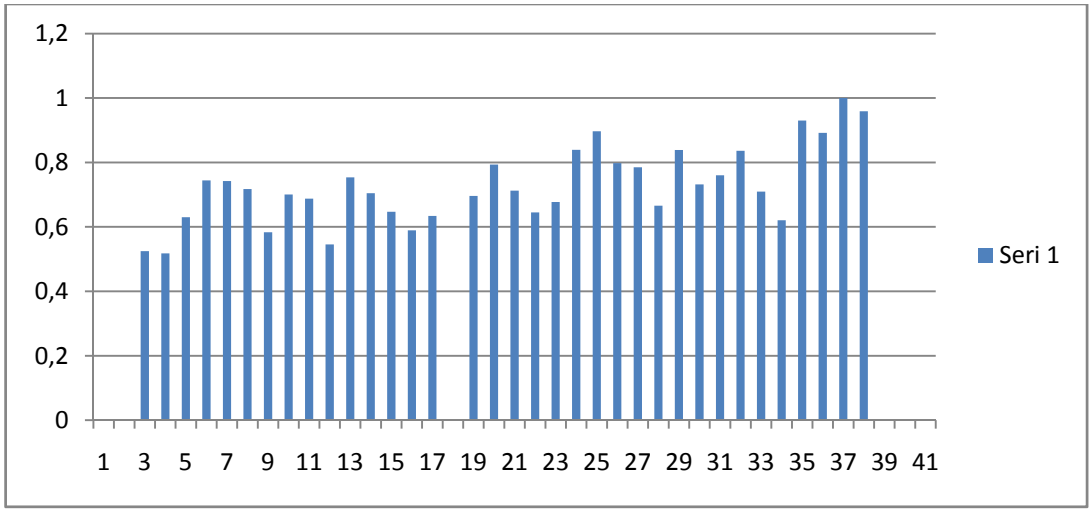
Seri 1

158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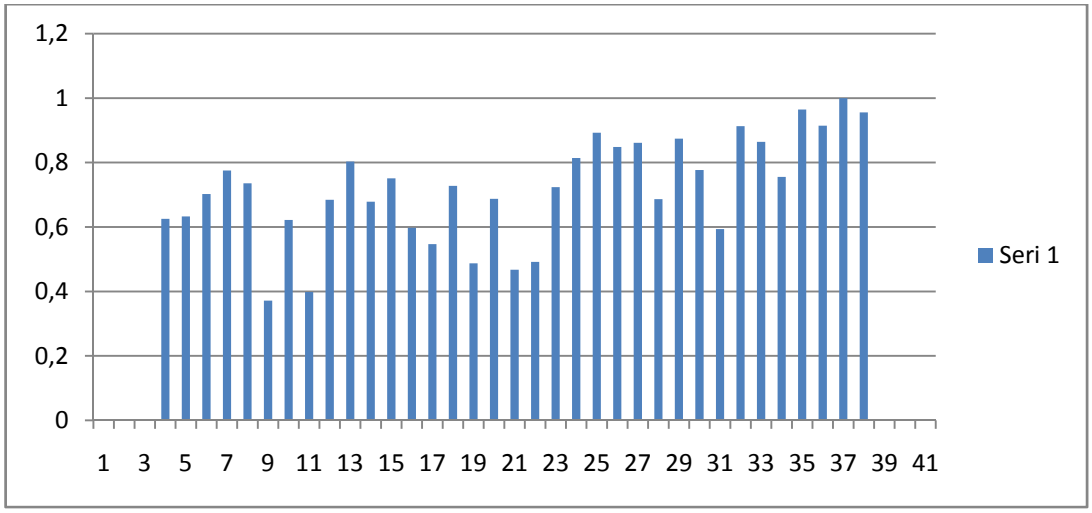
Seri 1

159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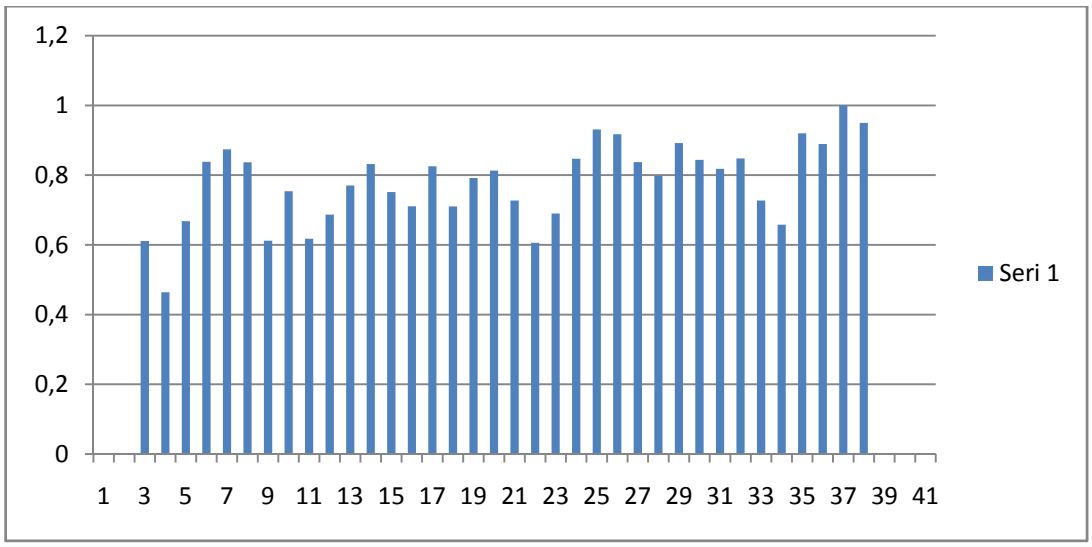
Seri 1

160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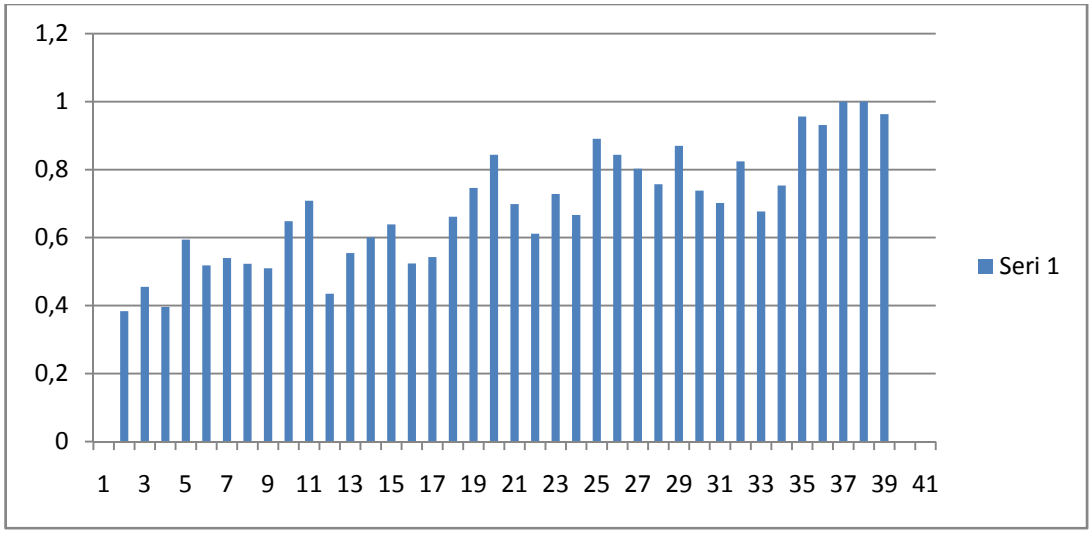
Seri 1

161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Seri 1

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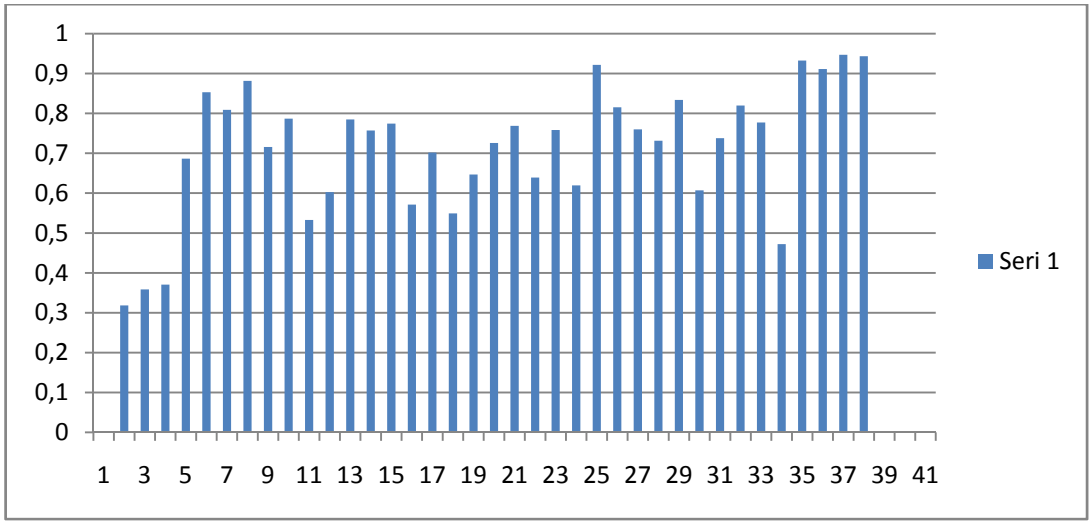
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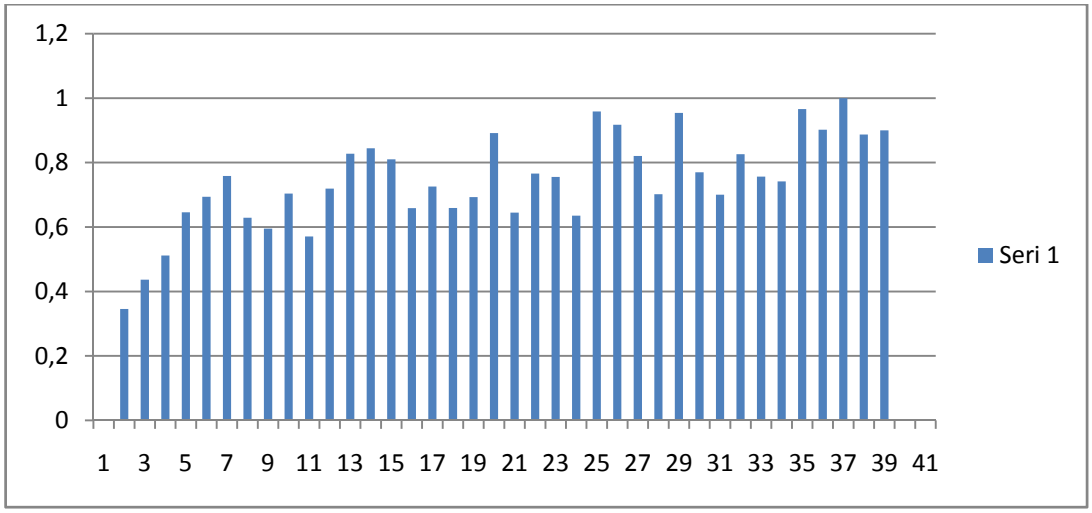
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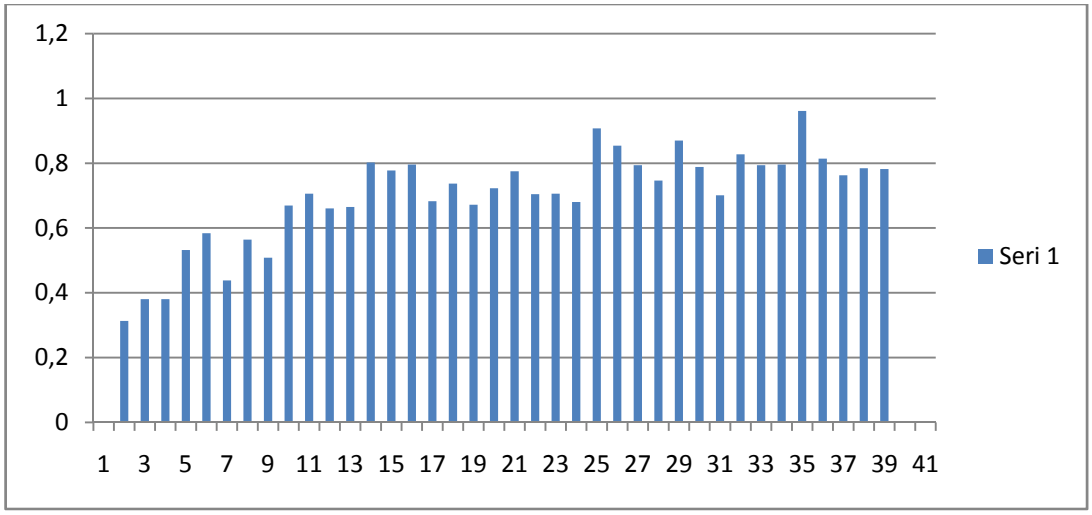
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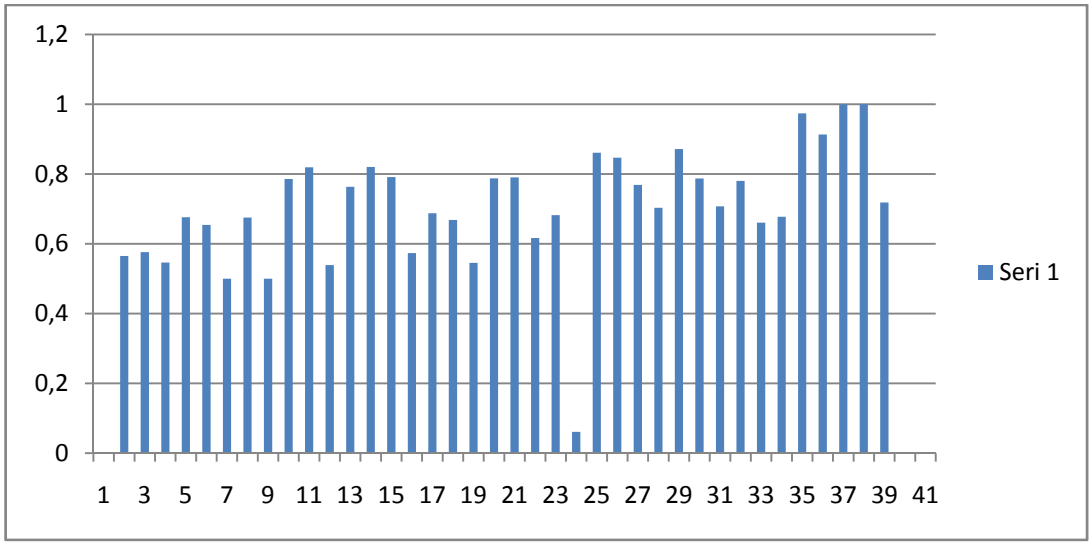
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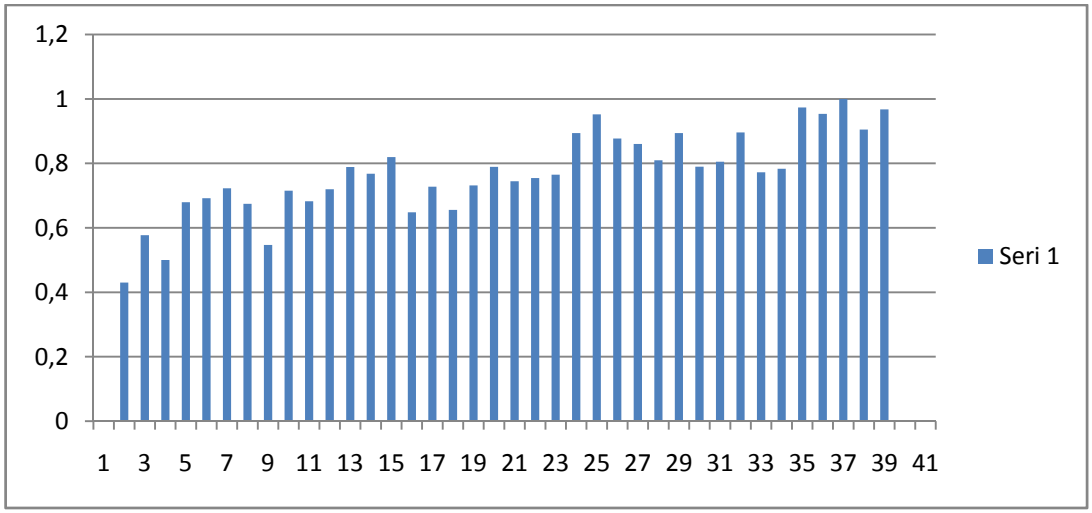
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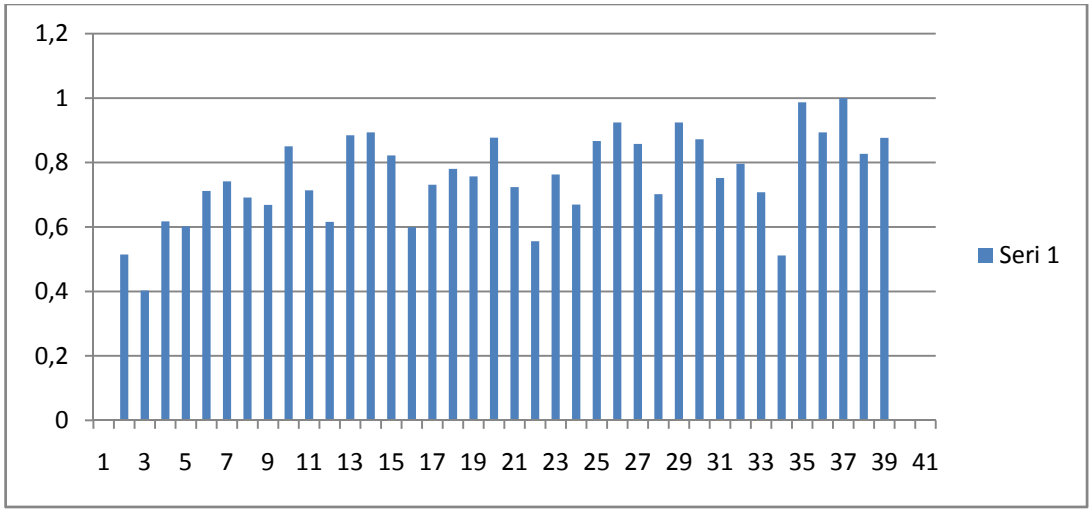
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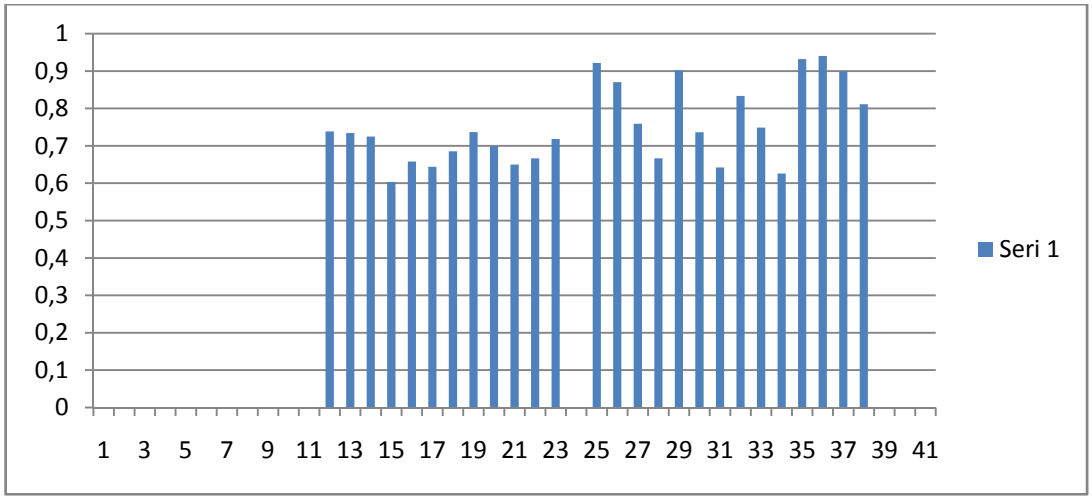
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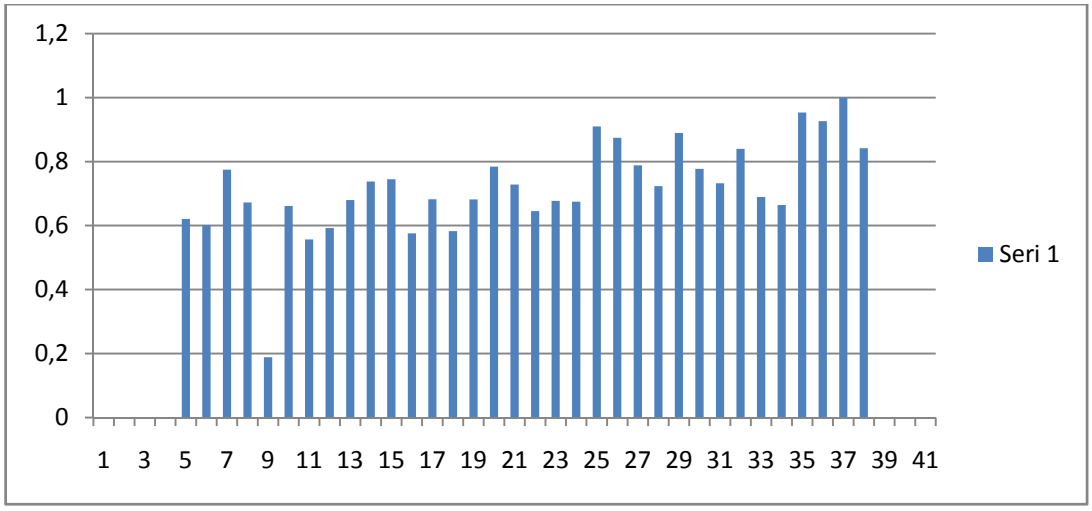
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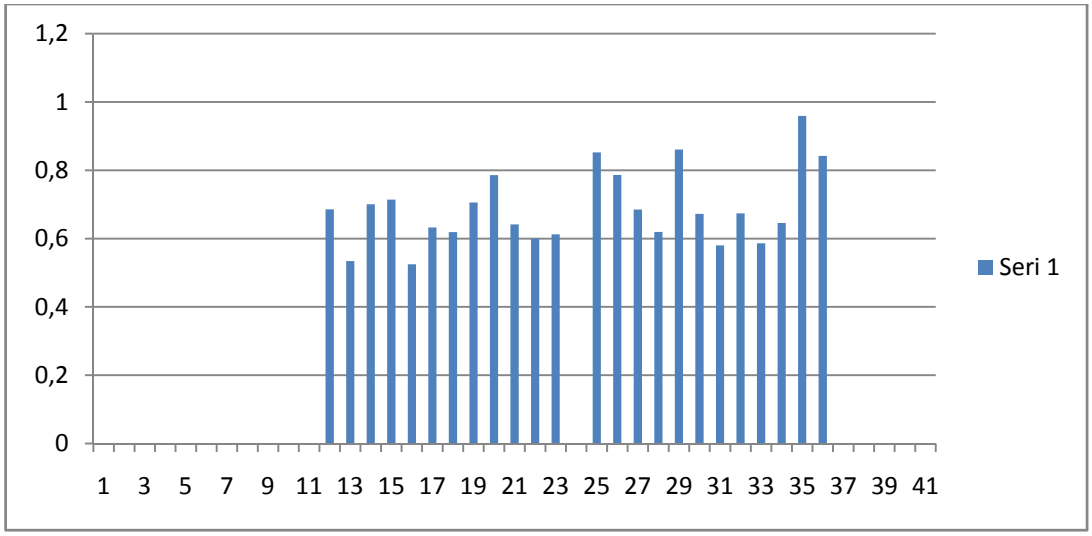
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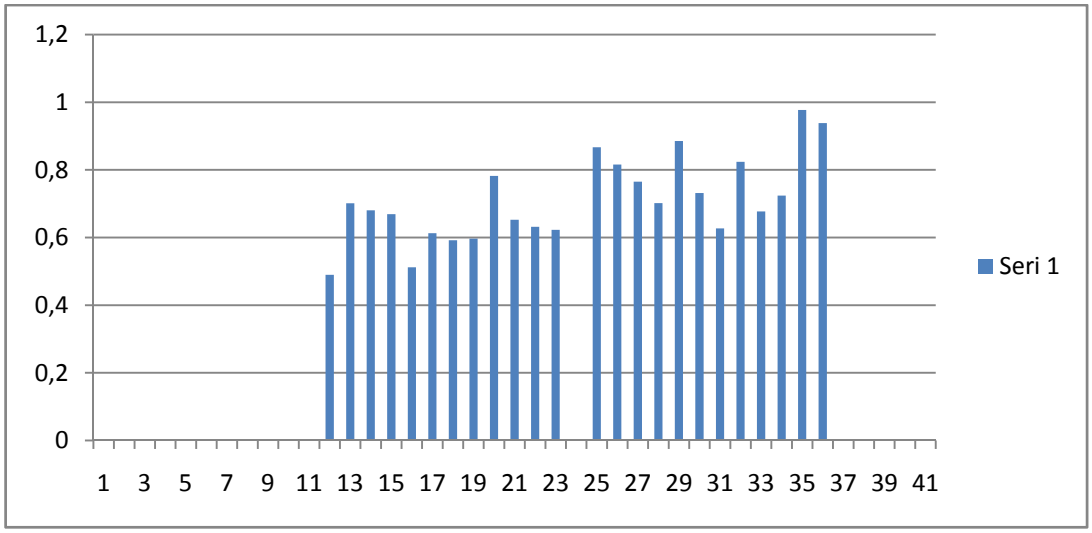
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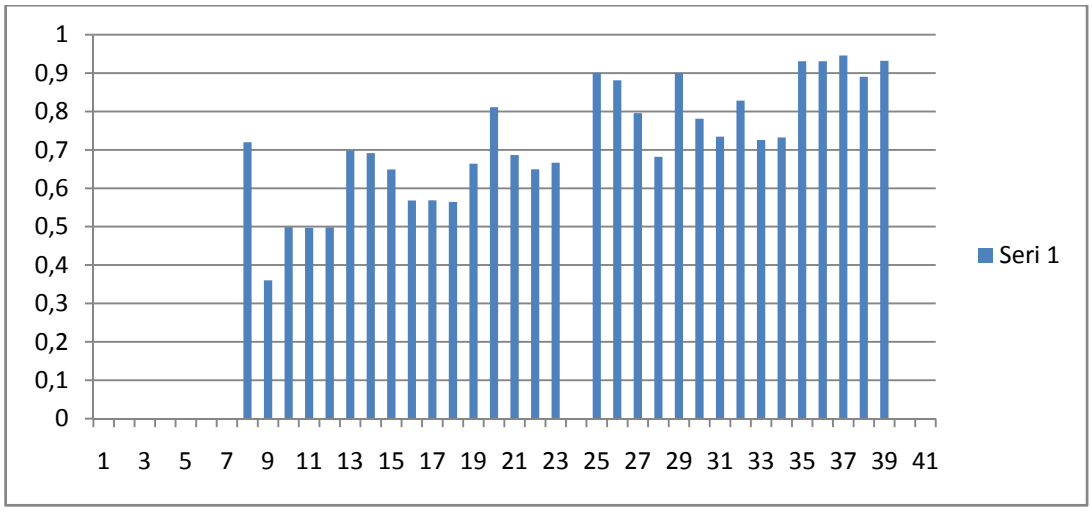
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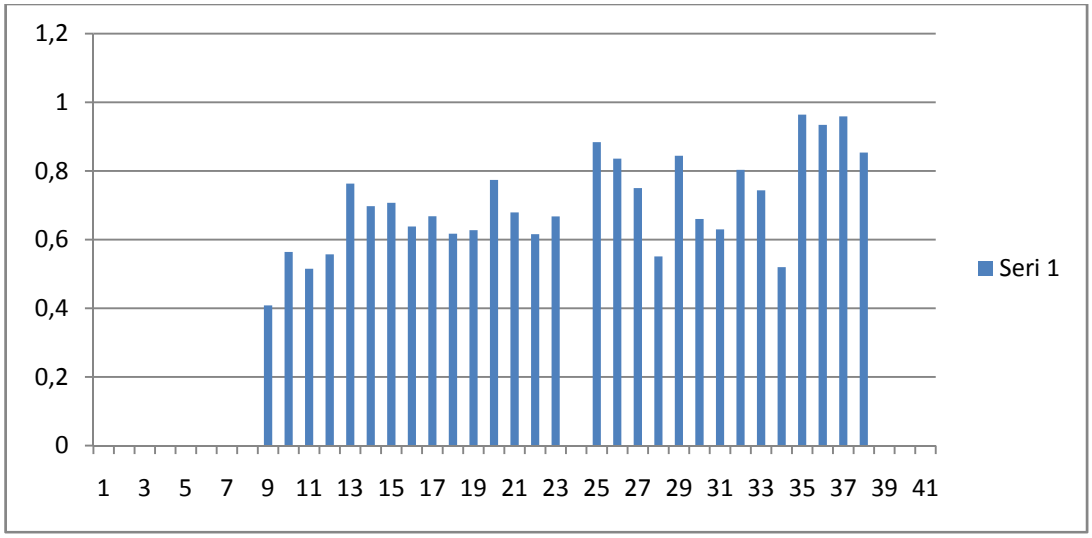
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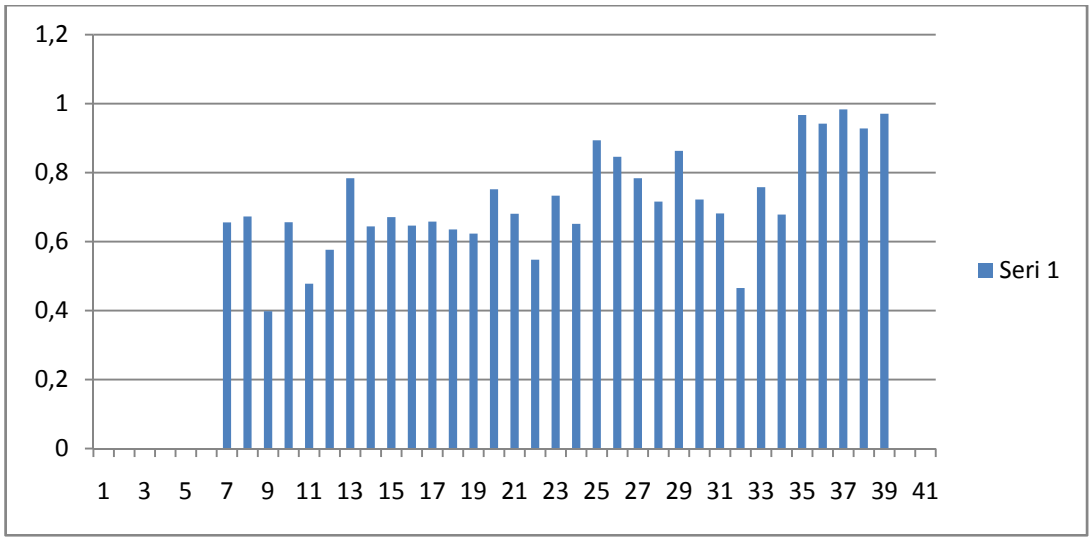
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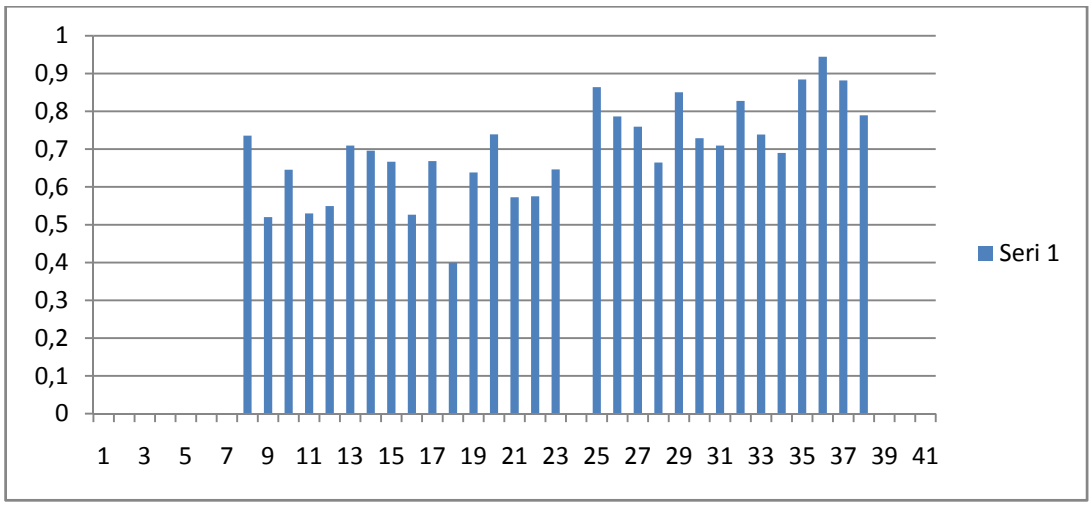
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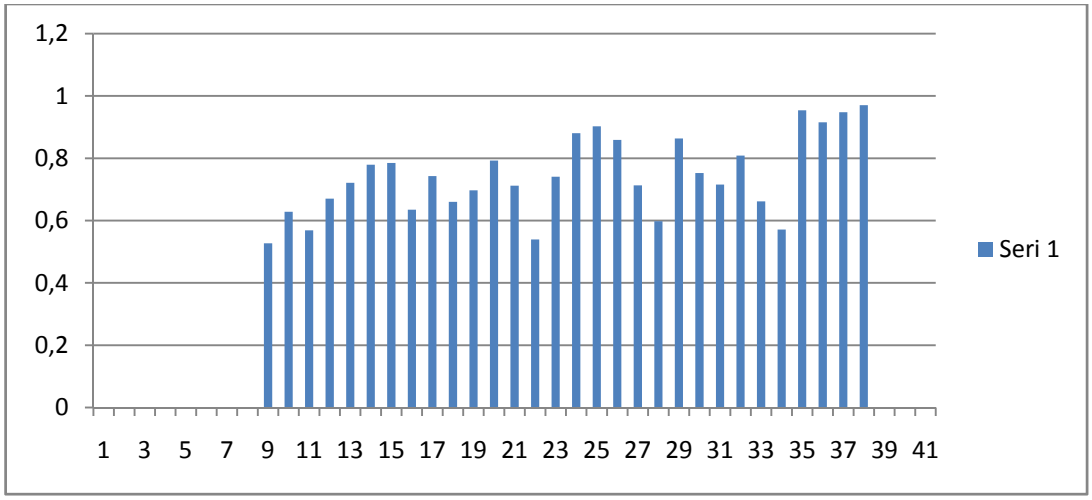
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CURRICULUM VITAE

Esra Karaca was born in Kayseri, in 1983. She enrolled to the Istanbul Technical University, Molecular Biology and Genetics Department in 2001. She graduated from the Molecular Biology and Genetics Department in 2006 and started to MSc degree education in Molecular Biology Genetics and Biotechnology Program of the same university.