

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**INVESTIGATION OF THE EFFECT OF CIRCADIAN CLOCK
DISRUPTION ON DNA METHYLATION**

MELTEM AŞICIOĞLU
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

SİRKADİYEN SAAT BOZULMASININ
DNA METİLASYONU ÜZERİNE ETKİSİ

MELTEM AŞICIOĞLU
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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GEBZE

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SUMMARY

The circadian clock is a biological oscillator with a ~24-hour period that helps organisms maintain various metabolic functions. Many biochemical pathways, such as the cell cycle and DNA repair, are controlled by the circadian clock. The abnormalities in these pathways are closely related to tumour development. However, the DNA methylation pathway is also important for cellular homeostasis and its breakdown is closely associated with cancer. Therefore, we investigated whether the disruption of the circadian clock affects the methylation profile of genes used as biomarkers in breast cancer.

In this study, we used two breast epithelial cell lines; MCF10A is non-tumorigenic and MDA-MB-231 is invasive. *Clock*, *Bmal1* and *Cryptochrome* genes were knocked out by the CRISPR technique. The knockout of genes was shown by western blot. After DNA isolation, all samples were subjected to the bisulfite conversion. Samples were amplified with *BRCA1*, *RASSF1A* and *RARβ* primers on qPCR. The methylation profiles were analysed by High-Resolution Melting (HRM). The circadian clock gene mutants were compared to their parental cell lines.

The circadian clock mutants exhibited the same methylation status as the parent cells. The knockout of the *Clock*, *Bmal1* and *Cryptochrome 1&2* genes was found to have no effect on the methylation status of the *BRCA1*, *RASSF1A* and *RARβ* genes.

The populations in both phases quenched each other because the biological clock in the wild-type cells used was not reset. The difference between the wild and knockout cells may not have been distinguished because this created the average knockout phenotype in the wild.

Keywords: Circadian Clock, Breast Cancer, DNA Methylation, High-Resolution Melting (HRM) Analysis

ÖZET

Sirkadiyen saat, organizmaya çeşitli metabolik işlevleri sürdürmede yardımcı olan ~24 saatlik bir zamana sahip biyolojik salınımdır. Hücre döngüsü, DNA tamiri gibi birçok biyokimyasal yollar, sirkadiyen saatinin kontrolü altındadır. Bu temel yolaklardaki anormallikler, tümör gelişimiyle yakından ilişkilidir. Öte yandan, hücresel homeostaz için DNA metilasyonu önemlidir ve anormal metilasyon kanserle yakından ilişkilidir. Buradan yola çıkılarak, tez çalışmasında sirkadiyen saat bozulmasının meme kanserinde biyolojik belirteç olarak kullanılan genlerin metilasyon profilini etkileyip etkilemediği araştırıldı.

Bu çalışmada, meme dokusundan kaynaklanan iki epitelyal hücre dizisi kullanılmıştır. MCF10A, farelerde tümör oluşturmeyen hücre hattı iken; MDA-MB-231, invaziv meme kanseri hücre hattıdır. Sirkadiyen saat genleri olan *Clock*, *Bmal1*, *Cryptochrome* genleri CRISPR tekniği ile susturuldu. Western blot analizi ile genlerin susturulduğu gösterildi. DNA izolasyonunun ardından, tüm örnekler bisülfid çevrimine tabii tutuldu. Örnekler BRCA1, RASSF1A ve RAR β primerleriyle qPCR’da çoğaltıldı. Metilasyon profilleri, Yüksek çözünürlüklü Erime Analizi (HRM) yöntemi ile analiz edildi. Sirkadiyen saat gen mutantları, ana hücre hatları ile kıyaslandı ve metilasyon profili değişimleri incelendi.

Tüm mutantlar, ana hücre hatları ile aynı metilasyon profilini sergilediği gözlemlendi. *Clock*, *Bmal1* ve *Cryptochrome 1&2* genlerinin ‘‘knockout’’ edilmesinin, *BRCA1*, *RASSF1A* ve *RAR β* genlerinin metilasyon durumları üzerine bir etkisi olmadığı bulundu.

Kullanılan yabancı tip hücrelerde biyolojik saatin resetlenmemiş olması sebebi ile her iki fazda bulunan popülasyonlar birbirinin etkisini söndürmektedir. Bu durumun yabancı hücrelerde ortalama ‘‘knockout’’ fenotipi oluşturmaları nedeniyle yabancı ve knockout hücre hatları arasındaki metilasyon farkının ayırt edilemediği düşünülmektedir.

Anahtar Kelimeler: Sirkadiyen Saat, Meme Kanseri, DNA Metilasyonu, Yüksek Çözünürlüklü Erime (HRM) Analizi

ACKNOWLEDGEMENTS

I would like to thank my advisors Assoc. Prof. Dr. Nuri ÖZTÜRK and Assoc. Prof. Dr. Müslüm AKGÖZ for their thoughtful guidance. It was a great chance for me to study with their experience and support provided me a valuable work experience. I would also like to thank to my jury members Prof. Dr. Tanıl KOCAGOZ and Prof. Dr. Sedef TUNCA GEDİK for spending their valuable times to evaluate this thesis.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
AngII	: Angiotensin
ATM	: Ataxia Telangiectasia-Mutated
ATR	: ATM and Rad3-related
bHLH	: Basic helix-loop-helix
BMAL1	: Brain-muscle-Arndt-like-protein-1
bp	: Base pair
BRCA1	: Breast cancer susceptibility1 gene
CCNA	: Cyclin A
CCNB	: Cyclin B
CCND	: Cyclin D
CCNE	: Cyclin E
CDKs	: Cyclin-dependent kinases
CK1 ϵ	: Casein kinase 1 epsilon
Chk2	: Check Point Kinase 2
CLOCK	: Circadian-locomotor-output-cycles-protein
CO ₂	: Carbon dioxide
CpG	: Cytosine phosphate guanine
CRY	: Cryptochrome
Cq	: Cycle of quantification
ddH ₂ O	: Double distilled water
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium Bromide
g	: Gram
HRM	: High Resolution Melting
IR	: Ionizing radiation
LAN	: Light-at-night
LB	: Luria Bertani
MCF10A	: Non-tumorigenic Human Epithelial Breast Cell Line
MDA-MB-231	: Tumorigenic Human Epithelial Breast Cell Line

MgCl ₂	: Magnesium chloride
min	: Minute
MLT	: Melatonin
M	: Molar
mM	: Milimolar
μM	: Micromolar
μg	: Microgram
μl	: Microlitre
PAS	: Period-Arnt-Single-minded
PCR	: Polymerase chain reaction
PER	: Period
pH	: Quantitative measure of the acidity or basicity of aqueous or other liquidsolutions (Power of Hydrogen)
Ppary	: Peroxisome proliferator-activated receptor
PRC	: Phase response curve
qPCR	: Quantative Polymerase chain reaction
RARβ	: The retinoid acid receptor-p
RASSF1A	: Ras association domain-containing protein 1
REV-ERBα	: V-erba-related protein
Ror-α	: Retinoic acid receptor alpha
RPM	: Revolution Per Minute
SCN	: Suprachiasmatic nucleus
sec	: Second
TBE	: Tris Boric Acid EDTA
TE	: Tris EDTA
TIM	: Timeless
Tm	: Melting Temperature
TTFL	: Transcription Translation Feedback Loop
XPA	: Xeroderma pigmentosum group A
ZT	: Zeitgeber time

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1. INTRODUCTION

The subject of this project is understanding the effect of disruption of circadian rhythm on the methylation of representative genes that are important for cancer development. Previous studies show that disruption of the circadian rhythm is highly related between cancer diseases in the literature. Circadian rhythms are ~24-hour cycles in metabolic or physiological events. Many molecular processes are controlled by circadian rhythm. Therefore, disruption of the circadian clock is expected to contribute to certain diseases, including epithelial cancers, such as breast cancer. Additionally, the epigenetic regulation of DNA is significant in gene expression and altered gene transcription. DNA methylation is most commonly part of epigenetic regulation. Aberrant methylation is observed in cancer cells, which lose differentiation and regulation. Therefore, many genes undergo hypermethylation or hypomethylation in cancer. In this thesis, we have investigated the relationship between circadian rhythm disruption and DNA methylation using the MCF10A and MDA-MB231 cell lines and their *Clock*, *Bmal1* and *CRY 1&2* knockouts.

1.1. Purpose, Contribution and Content of Thesis

We aimed to understand the effect of the knockout of circadian clock genes *Clock*, *Bmal1* and *CRY 1&2* on the methylation of the BRCA1, RASSF1A and RAR β genes, which are important for breast cancer development.

This study will contribute to the understanding of the circadian clock's effect on the methylation of certain genes that play roles in the development of breast cancer. To our knowledge, this is the first study that analyses the methylation in human breast cell lines in which circadian clock genes are modified by Cas9 Nickase mutant and Cas9 wild-type (lentivirus) CRISPR techniques. We expect that the methylation status of BRCA1, RASSF1A and RAR β will be especially important for the comparison of the CLOCK/BMAL1 and CRYPTOCHROME mutants because they regulate target genes in the opposite manner. The output of this thesis will reveal the direct effect of the molecular clock on the methylation of certain cancer-related genes that can be targeted for intervention for therapeutic purposes.

In Chapter 2, detailed information is given about circadian rhythms, DNA methylation and the relationship between the disruption of circadian rhythm and diseases. In Chapter 3, the materials and all experimental methods are described. In Chapter 4, the results are provided. The thesis ends with a discussion in Chapter 5.

2. LITERATURE REVIEW

2.1. Circadian Rhythm

Circadian rhythm is a biological process with a ~24-hour period that is maintained in stable conditions [Bell-Pedersen et al., 2005]. The Earth's rotation around its axis causes day–night periodicity, which affects the physiology of most living organisms, which are as diverse as cyanobacteria, fungi, algae, plants, flies, birds and mammals [Gery and Koeffler, 2010]. The word ‘circadian’ originates from the Latin words *circa* (about) and *dian* (day). These clocks are intrinsic and time-tracking systems that allow organisms to predict environmental changes (e.g., nutrient availability and predatory pressure) and adapt their behaviour and physiology to a favourable time of day [Sahar and Sassone-Corsi, 2009].

A variety of molecular, physiological, biochemical and behavioural processes, such as the sleep–wake cycle [rest–activity], metabolism and hormone secretion, are controlled by circadian synchronisation [Kelleher et al., 2014]. The rest–activity cycle is the best-known example of circadian rhythm. Numerous organisms exhibit similar behaviour, which is generally named the *rest–activity cycle*, to human sleep–wake cycles [Marcheva et al., 2013].

2.1.1. Endogenous Clocks Are Controlled by Circadian Rhythm

The periodicity of circadian rhythms under non-cycling environmental conditions alters between different species. The period in most situations did not certainly match that of any environmental cycle; therefore, this period was most likely generated endogenously. For example, activity begins each day and ends slightly earlier than it did the day before in constant darkness (Figure 2.1). This drift does not occur because light resets the circadian system [Sehgal, 2004].

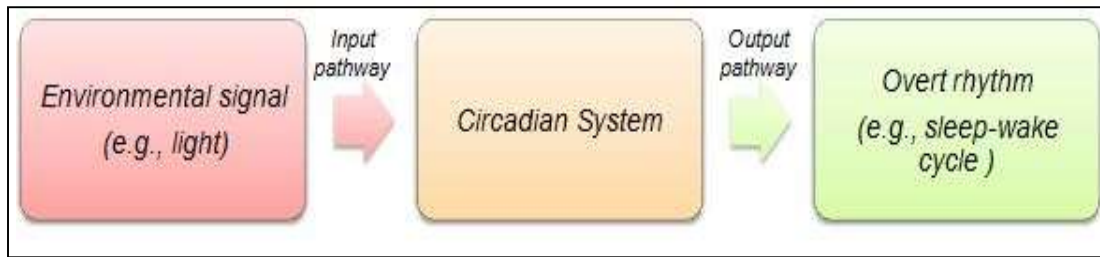


Figure 2.1: A simple circadian system.

Not all rhythmic functions are controlled by an endogenous clock. Some are guided by the day–night cycle and therefore do not require constant conditions. These are known as diurnal rhythms. Organisms that have diurnal rhythms maintain non-cycling environmental conditions [Sehgal, 2004]. In humans, rhythmic processes include sleep–wake cycles, the regulation of body temperature, blood pressure, urine production, cholesterol metabolism, respiratory function, incidence of asthma, heart attacks and secretion of several hormones. The release of some hormones is rhythmic since it is affected by sleep rather than clock function [Rana and Mahmood, 2010].

2.1.2. Input and Output of Circadian Rhythm

The circadian clock system contains input and output components. External/environmental cues known as the *input* or *entrainment pathway* entrain clocks [Sahar and Sassone-Corsi, 2009]. Light is a predominant entraining signal; therefore, the input pathway is generally comprehended as a photoreceptor. Physiology and behaviour are affected by time-of-day signals. Chronobiologists refer to the *output pathway*, which transports the signals from the clock and produces an overt rhythm. There must be most output pathways, because outputs are mostly diverse, even within the same living creature. Even though this system’s components are characteristically described in a linear diagram, this is an oversimplification of the mechanisms involved. In numerous statuses, outputs are able to feedback and influence the circadian system. For instance, the clock can be reset by forced activity. Input maybe regulated by clock feedback. The response to light becomes stronger at specific times of the day. This issue is called being ‘gated’ by the clock [Sehgal, 2004].

2.1.3. Multiple Clocks

Numerous multicellular organisms have more than one circadian clock. Just as physiological functional complexity increases, there is a rising necessity to have specific clocks for several organs and tissue systems [Sehgal, 2004]. Circadian complexity results from the molecular oscillator entity. These oscillators share the same molecular machinery in diverse cell types [Bell-Pedersen et al., 2009]. This means that there is one clock that commands all others in an organism. This clock is referred to as the *master clock* or *central clock*. The master clock is located in the brain (Figure 2.2 and 2.3). *Peripheral oscillators* are clocks in other portions of the body; these are called peripheral oscillators to differentiate them from the master clock. The autonomy of these oscillators is converted from gene to gene and from oscillator to oscillator [Sehgal, 2004].

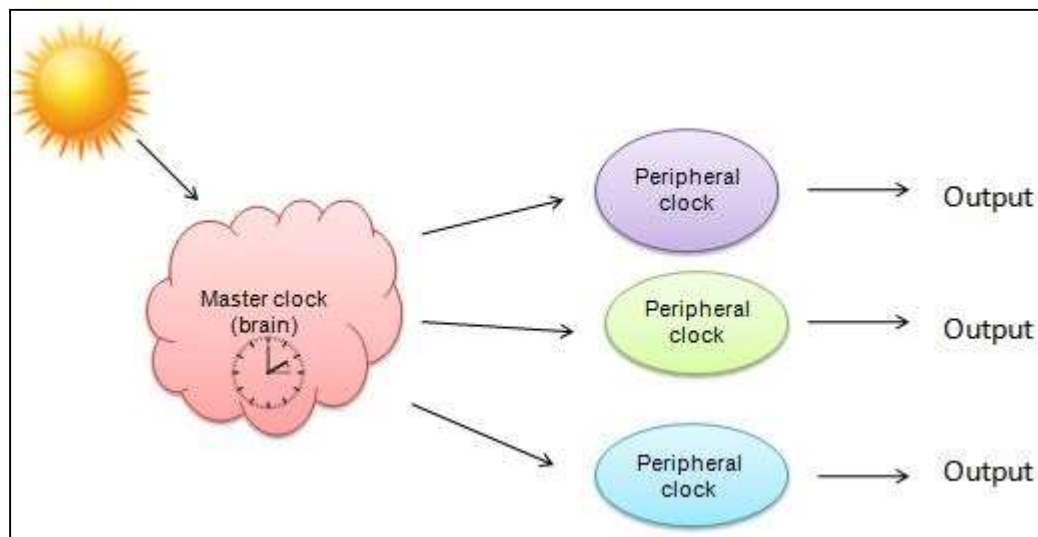


Figure 2.2: The master clock is located in the brain and affects peripheral clocks.

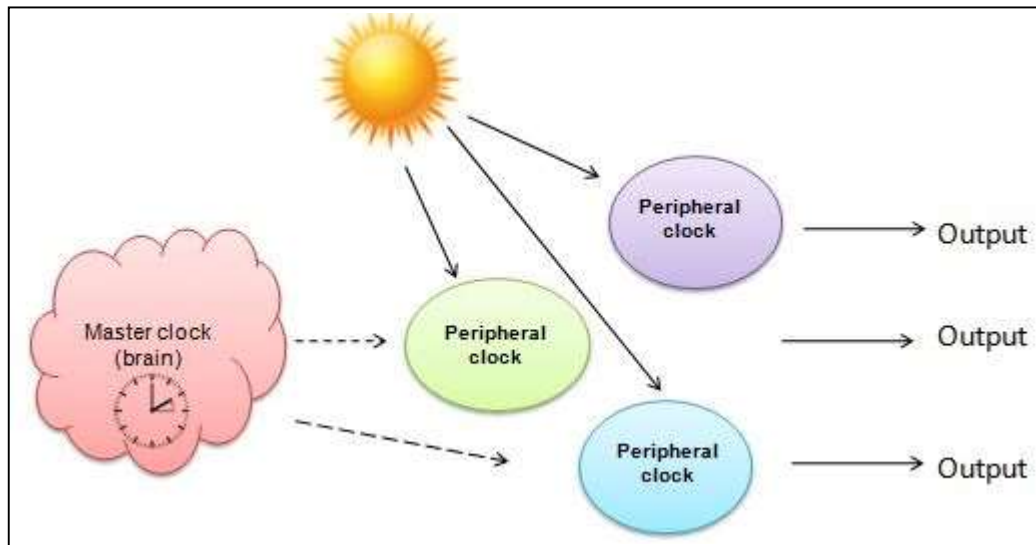


Figure 2.3: In mammals, peripheral clocks can be photosensitive. However, peripheral clocks can be entrained by other cues, such as hormones, even though they are not directly photosensitive.

2.2. Overview of Circadian Rhythm

Circadian clock system models have in widespread is the being of the feedback loop because overt rhythms must have their basis in a loop or endogenous rhythms that may maintain oscillation despite the lack of an environmental signal. A common idea for the clock mechanism was first studied in *Drosophila* and then in *Neurospora*. The clock function of mammal work is based on information obtained from other systems, particularly the *Drosophila* model [McClung, 2006].

The basic clock mechanism is a feedback loop that regulates the self-expression of the oscillating products of certain genes. These genes' autoregulation caused the negative regulation of their own mRNAs, and they are referred to as clock genes (Figure 2.4) [Bell-Pedersen et al., 2009].

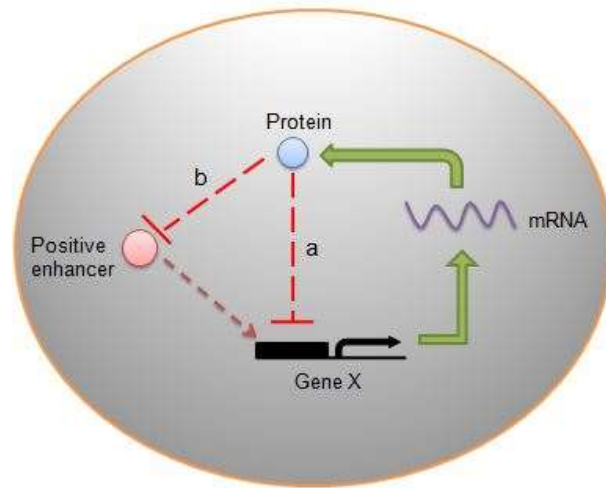


Figure 2.4: The basic model for circadian rhythm oscillation. The clock mechanism is conserved in numerous diverse species ranging from cyanobacteria to mammals. In a negative feedback loop in which a clock gene encodes mRNA and protein, this protein acts as the gene itself to downregulate its expression. This creates an oscillating pattern of gene expression. The loop takes approximately 24 hours to terminate. Gene expression inhibition is either direct (a) or indirect (b). When direct inhibition is caused by the protein inhibiting its own transcription, indirect inhibition occurs through the gene product inhibiting a positive element that leads to gene transcription.

In the rhythmic system, the nature of gene expression differs from one organ/tissue to another in the same organism as well as from species to species. While a gene may be expressed rhythmically in one organ or tissue, the same gene may not be expressed rhythmically in another organ or tissue. The reason for this is that a gene may play several roles in the other organ or tissue due to the fact that operates with which it is included does not round in the other organ or tissue [Sehgal, 2004].

Circadian rhythm controls many bodily functions. Approximately 5–9% of the genome is controlled by circadian rhythm. This has been shown in DNA microarray studies in mice [Akhtar et al., 2002]; [Panda et al., 2002]; [Storch et al., 2002].

Numerous gene promoter regions enclose elements recognised by transcription factors. Both clock genes and genes that are downstream of the clock result in rhythmic expression through the rhythmic activity of these transcription factors (Figure 2.4 and 2.5) [Dunlap, 1999].

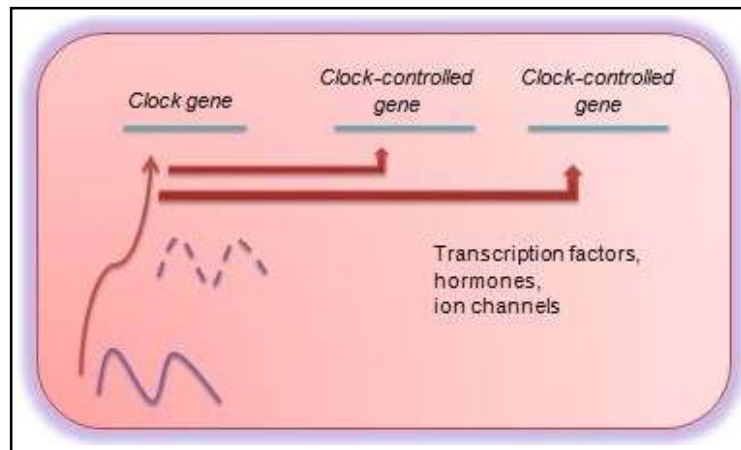


Figure 2.5: A molecular clock mechanism checks downstream genes. When the molecular clock regulates its own expression, its protein also regulates the downstream gene's transcription.

2.3. Mammalian Circadian Clock

2.3.1. The Master Clock in *Suprachiasmatic Nuclei*

Three components—the input, the output and the central oscillator—are comprised of the mammalian circadian clock. The core circadian clock manages a wide range of physiological and behavioural rhythms. The mammalian circadian clock is regulated in a hierarchy of oscillators. At the top of this hierarchy is the *suprachiasmatic nuclei* (SCN), which is the central oscillator located in the anterior part of the hypothalamus of the brain [Ko and Takahashi, 2006]. The SCN is composed of bilateral nuclei containing nearly 10,000 neurons each and controls independent peripheral oscillators [Takahashi et al., 2008]. This rhythm oscillates in an almost 24-h period even with a lack of environmental signals [Sukumaran et al., 2010]. In rodents, targeted lesioning of the SCN interrupts the circadian clock in locomotor activity, feeding and drinking [Stephan and Zucker, 1972]; [Moore RY, 1983]. However, surgically implanted SCN tissue is able to reconstruct rhythms with a period decided by the donor [M. R. Ralph et al., 2016]; [King VM, 2003].

Other circadian oscillators known as 'peripheral clocks' are located in other parts of the brain and other organs [Sukumaran et al., 2010]. The eye is responsible for light detection. Photoc signals from the retina are transmitted to the SCN directly through a neural pathway called the retinohypothalamic tract [RHT]. The SCN takes direct input

that entrains and synchronises the central clock to the external light–dark cycle [Savvidis and Koutsilieris, 2012]. The outputs of the SCN control the anterior and posterior pituitary hormones [Kriegsfeld and Silver, 2006b]. In addition to hormonal outputs, the hypothalamus and medulla area control the autonomic nervous system (Figure 2.6) [Buijs et al., 2006].

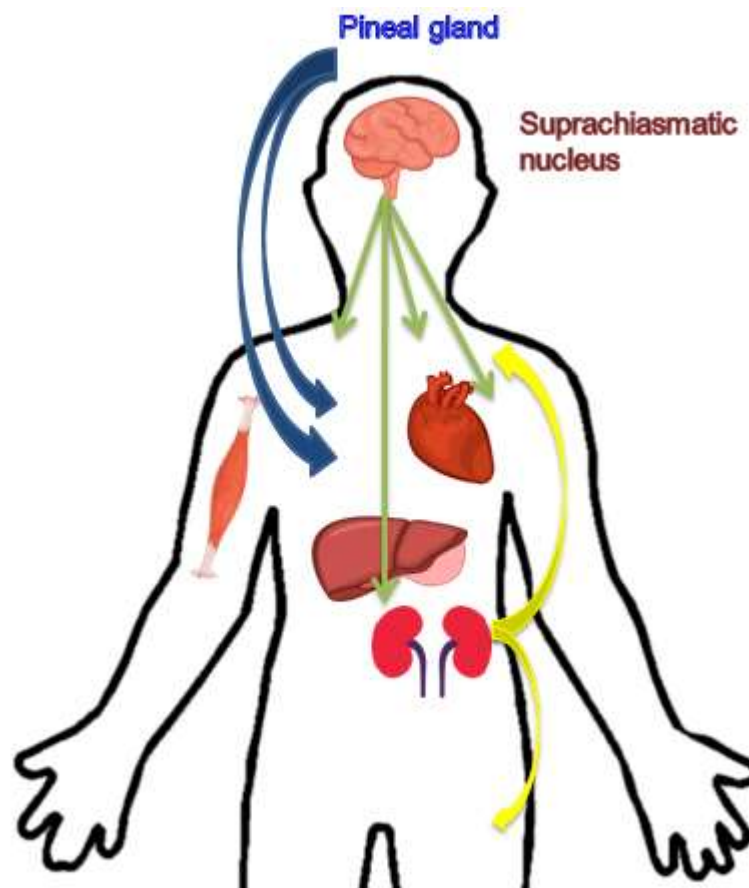


Figure 2.6: Pathways of peripheral clock entrainment. The master circadian pacemaker within the *suprachiasmatic nuclei* (SCN) relays interim information to peripheral oscillators. The dark blue arrows show the distribution of melatonin. This regulates temporal signals generated by the SCN. The green arrows demonstrate that circadian signals are emitted by nervous fibres from the SCN to the brain and peripheral targets. The yellow arrows indicate glucocorticoid synthesis. It is synthesised by the adrenal gland and secreted primarily around activity onset (dawn and dusk in diurnal and nocturnal species, respectively). Another hormonal output of the SCN is the daily rhythm of plasma glucocorticoids. They also distribute interim signals generated by the SCN to many tissue targets that express glucocorticoid receptors.

2.3.2. The Central Oscillator is Composed of a Feedback Loop

In mammals, the circadian clock is composed of a transcription–translation feedback loop (TTFL) [Ye et al., 2011]. Circadian oscillations consist of a group of genes that form a TTFL. These contain *Clock* (*Npas*), *Bmal1*, *Per* (*Per1* and *Per2*) and *Cry* (*Cry1* and *Cry2*). The BMAL1 and CLOCK proteins, members of the basic helix-loop-helix [bHLH]-PAS (Period-Arnt-Single-minded) transcription factor family, are positive elements. During the day, CLOCK and BMAL1 make a heterodimer that initiates the transcription of target genes containing E-box (5'-CACGTG-3') in the promoters of the *Per* and *Cry* genes, resulting in high levels of these transcripts (positive arm). PER and CRY make a heterodimer, translocate to the nucleus and interact with the CLOCK-BMAL1 complex, which results in inhibiting their own transcription (negative arm). During the night, the PER-CRY repressor complex is degraded and CLOCK-BMAL1 can become active to a new cycle of transcription again [Takahashi et al., 2008]; [Hall and Friedmann, 2011]; [Ko and Takahashi, 2006].

Researchers have indicated that another regulatory loop is induced by the CLOCK:BMAL1 heterodimers, such as retinoic acid-related orphan nuclear receptors Rev-erb α and Ror α [Mohawk et al., 2012]. REV-ERB α and ROR α subsequently compete to bind the *Bmal1* promoter present in retinoic acid-related orphan receptor response elements [ROREs]. This means that the ROR [α , β and γ] and REV-ER β [α and β] members can regulate *Bmal1* through ROREs. REV-ERBs repress the transcription of *Bmal1*, while RORs activate the transcription of *Bmal1*. Therefore, the circadian oscillation of Bmal1 is both positively and negatively regulated by RORs and REV-ERBs (Figure 2.7) [Ko and Takahashi, 2006]. Table 2.1 shows a list of important features at the molecular level.

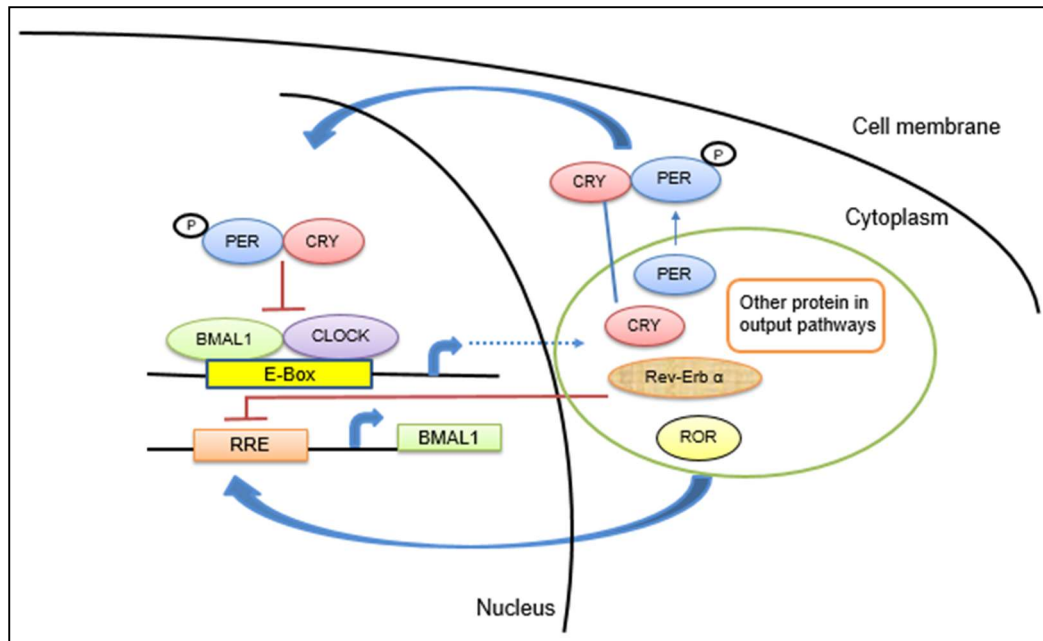


Figure 2.7: A transcriptional–translational feedback loop network. After the BMAL1 and CLOCK transcription products translocate to the cytoplasm and dimerise, they return to the nucleus and bind to the E-box regions. This binding promotes PER and CRY transcription. PER and CRY translocate to the cytoplasm and dimerise. Then, they return to the nucleus and inhibit the binding of the CLOCK/BMAL1 complex. Subsequently, PER and CRY are degraded. When ROR promotes *Bmal1* expression, REV-ERB α inhibits the transcription of *Bmal1*.

At a post-transcriptional level, the stability of the PER and CRY proteins is regulated by SCF [Skp1-Cullin-F-box protein] E3 ubiquitin ligase complexes. The kinases, casein kinase 1 ϵ/δ (CK1 ϵ/δ) and AMP kinase (AMPK), phosphorylate the PER and CRY proteins. This phosphorylation promotes polyubiquitination. The PER and CRY proteins are tagged for degradation by the 26S proteasome complex [Mohawk et al., 2012].

Table 2.1: Circadian Clock Genes; Roles, Products and Regulation

System	Gene	Clock Role	Protein Product(s)	Regulation	Phenotypes of Mutants
Mouse	Per1	(putative) negative elements	contains PAS domains which mediate interaction with other mammalian PER proteins; significance of interactions with negative element. TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT4 in SCN, around CT10 in retina and peripheral tissues; transiently induced by light	no mutants available
	Per2	(putative) negative elements	contains PAS domains which mediate interaction with other mammalian PER proteins; significance of interactions with negative element. TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT8 in SCN, around CT10 in retina and peripheral tissues; transiently induced by light	no mutants available
	Per3	(putative) negative elements	contains PAS domains which mediate interaction with other mammalian PER proteins; significance of interactions with negative element. TIM controversial; molecular relative of insect per	clear CR in mRNA expression with peak around CT6 in SCN, a broad peak ~CT10-14 in retina and peripheral tissues	no mutants available
	Tim	facilitating (negative?) element	clear sequence homolog of insect TIM	not rhythmically or weakly rhythmically expressed	no mutants available
	Clock	positive element	transcription factor: bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with BMAL1/MOP3; molecular relative of insect dCLK	not rhythmically expressed	one allele with long period length; homozygote has very long period, grading to ARR; reduced light induction of Per1
	Bmal1/mop3	positive element	transcription factor: bHLH DNA-binding domain, GLN-rich activation domain; PAS domains mediate heterodimerization with CLOCK; molecular relative of insect CYC	rhythmically expressed in rats; may not be rhythmically expressed in mice	no mutants available

2.4. Circadian Rhythm and Metabolism

2.4.1. Relationship between Circadian Rhythms, Cell Cycle and Apoptosis

The cell cycle, which governs cell division, consists of two critical phases: S phase and M phase. While the cell replicates its DNA in S phase, it divides (mitosis) in the M phase. The cells are preceded by growth phases, G1 and G2, and non-dividing (somatic) cells exist in a quiescent state (G0) [Feillet et al., 2015]. Events such as grow, divide or die, which depend on the cell cycle, have a specific phase, and cells appear to ‘know’ how to estimate them by using an intrinsic timing system [Hunt and Sassone-Corsi, 2007]. The molecular circadian clock system shares some similarities with the cell cycle. Firstly, both circadian rhythm and cell division are intracellular ‘clocks’. This means that both rely on sequential phase transcription–translation and protein modification and degradation. Secondly, both are based on the conceptual

device of interlocked autoregulatory loops [Hunt and Sassone-Corsi, 2007]. The clock genes lead to the rhythmic expression of up to 10% of all mammalian mRNA transcripts in diverse tissues [Panda et al., 2002]. Clock genes participate in some metabolic and physiological rhythms in diverse tissue, such as regulation of the cell cycle. The cell cycle depends on the transient and sequential activation of cyclin-dependent kinases (CDKs) that form complexes with cyclins (CCN). The progression of the cell cycle depends on CCND/CDK4–6 (G1), CCNE/CDK2 (G1/S transition), CCNA/CDK2 (S), CCNA/CDK1 (S/G2 transition) and CCNB/CDK1. These enzymatic complexes regulate cell cycle duration, especially at critical checkpoints [Feillet et al., 2015]. Many cell-cycle genes included in either G1-S or G2-M transitions involve E boxes in their promoters [Hunt and Sassone-Corsi, 2007].

Wee1, whose promoter involves three E boxes, is under the direct control of the CLOCK: BMAL1 heterodimer. The product of *Wee1* is a protein kinase that prevents the transition from G2 to mitosis by inactivating the phosphorylation of complex CDC2/CyclinB1 (Figure 2.8) [Matsuo et al., 2003]. In mammals, CLOCK heterodimerises with BMAL1 protein and the dimer promotes the expression of *cMyc* and *p21*. They produce proteins that play a significant role in proliferation and apoptosis. Additionally, the rhythmic expression of *Bax* [proapoptotic] and *Bcl2* [antiapoptotic] genes is under the control of clock oscillation. Moreover, the dimer CLOCK:BMAL1 joins the activation of *p53*, a proapoptotic gene. P53 protein promotes apoptosis in healthy cells if they are exposed to a DNA-damaging agent or initiates oncogenic transformation. Therefore, P53 protein plays a significant role in tumour suppression [Kramer and Merrow, 2013]. In the absence of p53, p73 can take the place of p53 as a tumour suppressor. As a result of the increased induction of p73 in cancer cells with both *Clock* and *p53* silencing [*Cry1*^{-/-} *Cry2*^{-/-} *p53*^{-/-}], apoptosis was increased. This evidence demonstrates a possible therapeutic role for *Cryptochrome* silencing in cancer cells with P53 mutation [Lee and Sancar, 2011].

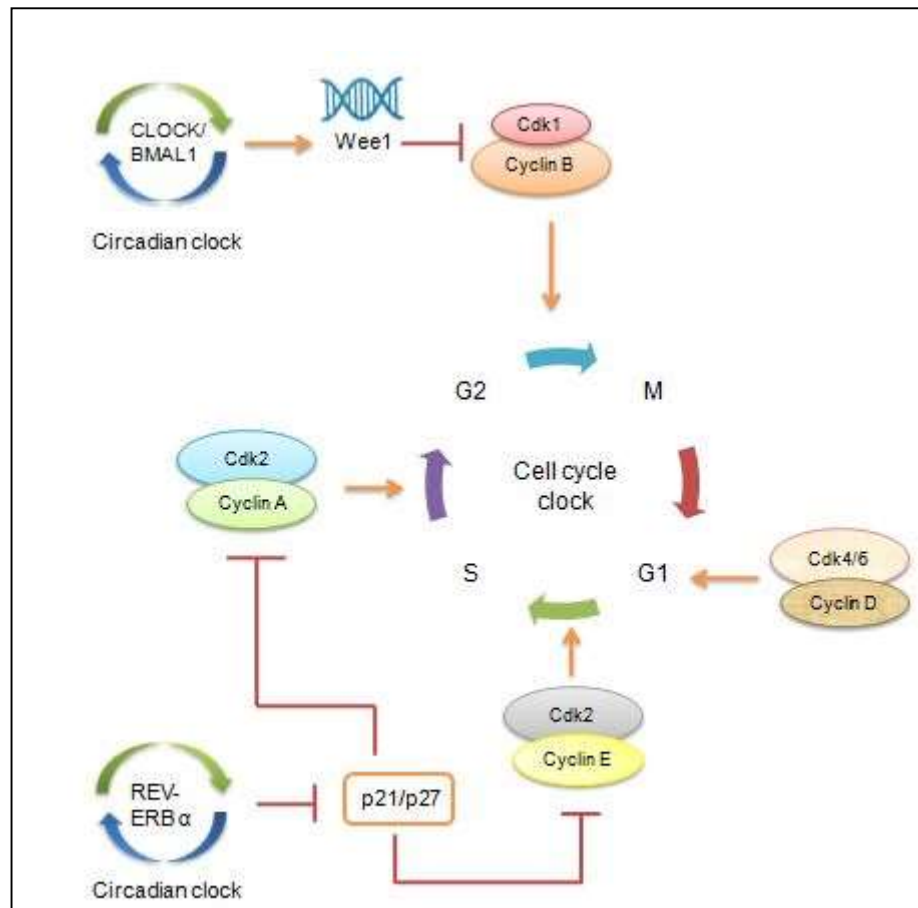


Figure 2.8: Circadian oscillation regulates cell cycle progression in multiple ways. Wee1 expression is stimulated by the CLOCK:BMAL1 complex. p21/p27, the inhibitor of Cdk, is repressed by REV-ERB α .

2.4.2. Relationship between Circadian Rhythms and DNA Damage Response

Organisms have developed various DNA-repair pathways and DNA-damage checkpoints to overcome the numerous challenges of endogenous and exogenous DNA damage. If there were no repair or checkpoint mechanisms, the organism's genome integrity would be frequently compromised [Hakem, 2008].

There are five principle pathways for DNA repair: direct repair, base excision repair, nucleotide excision repair, mismatches repair and recombination/cross-link repair. These repair mechanisms are almost universal. Pyrimidine dimers are repaired by nucleotide excision repair in mammals because mammalian organisms do not have photolyase. While the circadian clock controls nucleotide excision repair directly, it controls mismatch repair and double-strand break/cross-link repair indirectly [Sancar et al., 2015].

DNA damage sensing and DNA repair are regulated in part by xeroderma pigmentosum group A (XPA) [Kang et al., 2010]. XPA is a critical protein included in DNA damage recognition and a rate-limiting factor in excision repair. It is controlled by the circadian clock [Gaddameedhi et al., 2011].

Additionally, Ataxia Telangiectasia-Mutated (ATM) and ATM and Rad3-related (ATR) protein kinases are the two main DNA damage checkpoint pathways. The ATM pathway is induced by double-strand breaks formed by ionising radiation (IR) and other agents. Despite this, the ATR pathway is activated by bulky base adducts formed by UV and UV-mimetic agents [Sancar et al., 2015].

Gery and colleagues observed that PER1 interacted with ATM. ATM phosphorylated the cohesion of SMC1, p53 and the checkpoint kinase Chk2. Chk2 was also found to be associated with PER1-ATM. PER1 antagonised the cell division and function of CLOCK:BMAL1. While ectopic PER1 diminished the Wee1, Cyclin B1 and *cdc2* expression levels, *Per1* gene expression was downregulated in human tumours [Gery et al., 2006].

In addition, TIM is the other major molecular clock core protein, functionally interacting with ATM and ATR. While TIM interacts with Chk2, PER1 interacts with Chk1. ATM and ATR phosphorylate and activate the effector kinases Chk1 and Chk2, respectively (Figure 2.9) [Hunt and Sassone-Corsi, 2007].

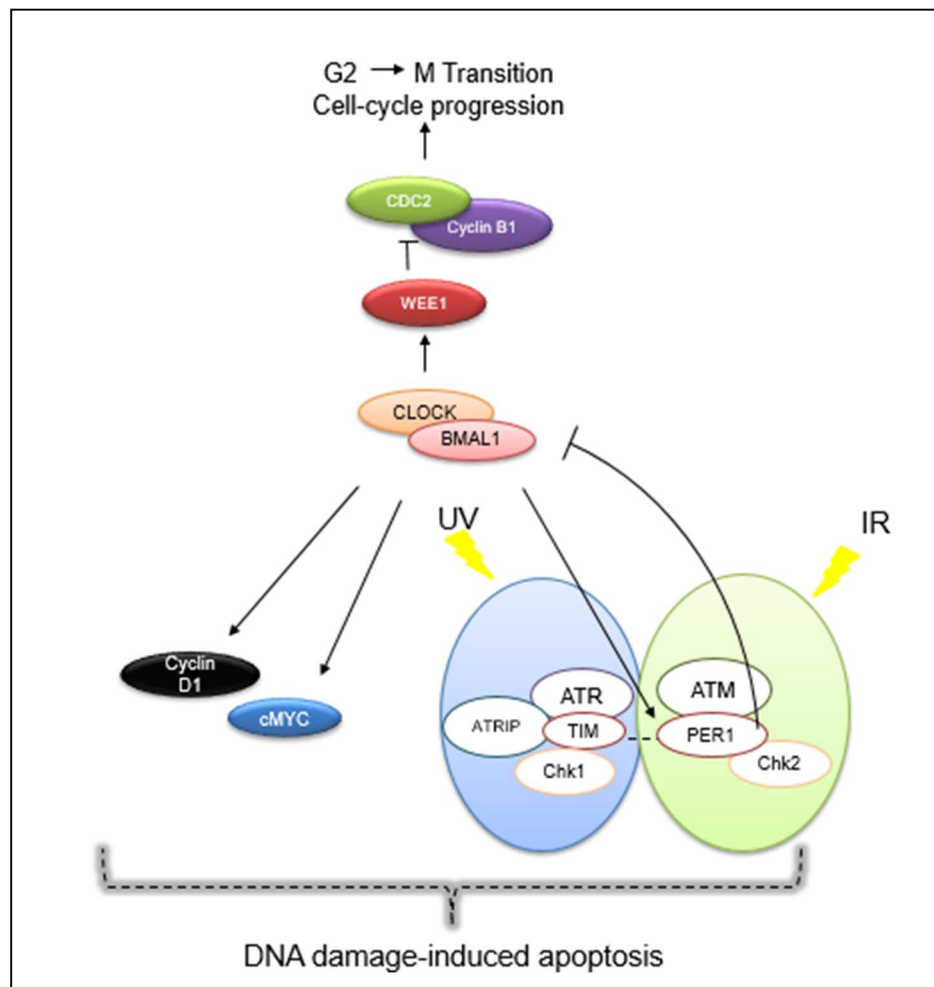


Figure 2.9: Molecular clock core genes and cell-cycle genes are induced by the CLOCK: BMAL1 heterodimer. The production of WEE1 kinase may activate the control of the G2-M transition and CDC2/Cyclin B1 complex. Encoding Cyclin D1 and c-Myc may also lead to the transition of the cell cycle. PER1 and TIM are probably related in the DNA-damage response. Both of them can be found complexed with the ATM and ATR kinases and the checkpoint kinases Chk2 and Chk1, respectively.

2.4.3. Relationship between Circadian Rhythms and Hormone Release Regulation

Circadian rhythm directly controls many biological and metabolic processes, such as daily rhythm in sleep/wake (or feeding/fasting) behaviour and regulating hormone release [Kramer and Mellow, 2013]. In the brain, the SCN drives hormone secretion through different pathways. While SCN neurons make synaptic connections with neurosecretion in some examples, SCN signals set the phase of clock genes that regulate circadian function at the cellular level within neurosecretory cells in other examples. SCN signals endocrine glands through the autonomic nervous system.

Therefore, the circadian clock accomplishes temporal regulation of endocrine function via a combination of genetic, cellular and neural regulatory mechanisms [Kriegsfeld and Silver, 2006a].

Prior to the oestrus cycle, oestrogen levels increase. This event promotes the production of brain progesterone receptors and releases a preovulatory surge in luteinizing hormone (LH). The regulation of progesterone receptor relative to oestrogen provides the time of ovulation [Hole et al., 1979]. After ovulation, progesterone secretion is essential for pregnancy; this is under the circadian control [Egli et al., 2007].

Plant and colleagues demonstrated that male rhesus macaques at 20-min intervals in an LD cycle revealed diurnal rhythms in LH, testosterone, prolactin, and cortisol [Plant, 1981]. In addition, investigations in humans indicate circadian alteration in gonadotropins and gonadal steroids around the onset of puberty [Jakacki et al., 1982]. Some of human testes' roles wax and wane over the years; for instance, in semen quality, the lowest sperm concentration is experienced from July to September [Levine et al., 1990]. Both semen quality and testosterone levels are highest in late fall and winter [Dabbs, 1990]. It has been demonstrated that diurnal alteration in hormone concentrations may simply be modulated by sleep.

Melatonin (MLT) is an efferent endocrine output of the SCN. It ensures a circadian message to the organism via the common cycle. The opposite of corticosterone, it is only affected by the light/dark cycle [Pevet and Challet, 2011]. While the melatonin content is low during the day, its content is high during the night [Ralph et al., 1971].

Humans are diurnal organisms whose circadian rhythm relies on natural light/dark cycles, but the invention of the electrical light bulb provided more opportunities for work and leisure. However, exposure to artificial light at night [LAN] affects our biological clock and suppresses pineal melatonin production [Haim and Portnov, 2013]. Artificial light during the night causes misinformation to be sent to the SCN. As a result of this process, pineal melatonin metabolism is disrupted. The disruption of the melatonin cycle contributes to chronodisruption and pathophysiology [Reiter et al., 2011].

2.5. Circadian Rhythm Disruption and Disease

Shift work, rapid travel across time zones, illness or advanced age can disrupt the circadian rhythmicity. When the sleep–wake cycle is out of phase with the circadian clock which governs rhythms, human behaviour, physiology, performance, safety, productivity and health can all be negatively affected. This is also related to many mental and physical disorders [Adler et al., 2001]. Some studies have revealed that reduced sleep duration and poor-quality sleep are associated with damaged glucose tolerance, decreased insulin responsiveness following glucose challenge, ascended body mass index, reduced levels of leptin, and increased levels of ghrelin [Donga et al., 2010]; [Gottlieb et al., 2005]; [Megirian et al., 1998]; [Nilsson et al., 2004]; [Taheri et al., 2004]; [Knutson and Cauter, 2008]; [Spiegel et al., 2009].

2.5.1. Diabetes

The first relationship between circadian rhythm and disease was found in mice carrying the *Clock*^{*Δ19/Δ19*} mutation. These mice were exposed to continual darkness. After that, they became arrhythmic and showed hyperphagia, hyperlipidaemia, hyperleptinaemia, hepatic steatosis, and hyperglycemic hypoinsulinaemia because of disrupted insulin secretion [Marcheva, Ramsey, and Bass, 2011][Turek et al., 2013]. Additionally, animals carrying the *Clock*^{*Δ19/Δ19*} mutation demonstrated a disruption of glucose and lipid homeostasis in the pancreas, liver and muscle because they had lost the rhythmic expression of metabolic and proliferative genes [Marcheva et al., 2010]; [McCarthy et al., 2007]; [Miller et al., 2007]; [Panda et al., 2002].

Scherr and colleagues studied healthy volunteers who were placed on a 28-hour ‘day’ and were organised to sleep at various phases during their circadian period. Volunteers showed ascended glucose, higher blood pressure and reduced leptin when shifted 12 hours from their normal sleep/wake cycle; their post-meal glucose response was similar to that seen in prediabetic patients [Scheer et al., 2009].

In mice, both *Clock* gene mutations and *Bmal1* gene mutations trigger arrhythmia. While *Clock*-deficient mice can generate normal behavioural and molecular rhythms, the null allele of *Bmal1* has severely disrupted behavioural and

molecular rhythms. However, both the disruption of *Per1* and *Per2* genes and *Cry1* and *Cry2* genes trigger behavioural and molecular arrhythmia. For example, *Bmal1*-deficient mice have diverse phenotypes such as the loss of circadian rhythms and body weight, shortened lifespan, infertility, progressive arthropathy, abnormal gluconeogenesis [Ko and Takahashi, 2006]. Moreover, loss of BMAL1 also damages adipogenesis, adipocyte differentiation and hepatic carbohydrate metabolism [Lamia et al., 2011]; [Rudic et al., 2004]; [Shimba et al., 2005]. Some studies have revealed that peripheral tissue-specific circadian rhythms have different functions within pancreatic islets and the liver. The deletion of pancreas-specific *Bmal1* leads to impaired β -cell proliferation and insulin granule exocytosis. This also causes hyperglycaemia, the disruption of glucose tolerance, and diminished insulin response. It is unlikely that the disruption of liver-specific *Bmal1* can cause a loss of the oscillation of key hepatic metabolic genes, impaired gluconeogenesis, exaggerated glucose clearance, and hypoglycaemia during the resting phase [Lamia, Storch, and Weitz, 2008]; [Marcheva et al., 2010]; [Sadacca et al., 2011]. These all indicate that *Bmal1* has a role in diverse functions in the tissue type in which it is expressed. In contrast, *Clock*-deficient mice have mild effect circadian phenotypes in a gene-specific and tissue-specific attitude [Ko and Takahashi, 2006]. The studies using *Clock* gene mutants show characteristics of metabolic syndrome such as hyperleptinaemia, hyperlipidaemia, hepatic steatosis, hyperglycaemia and hypoinsulinaemia.

Studies in mice mutant for *Cry1* and *Cry2* have shown that these animals developed glucose intolerance, high corticosterone levels, ascended glucocorticoid transactivation in liver, changed lipogenic and steroidogenic pathways, and disrupted body growth and liver regeneration [Bur et al., 2009]; [Lamia et al., 2011]; [Okamura, Doi, Yamaguchi, and Fustin, 2011]; [Matsuo et al., 2003]. Similarly, Zhang and colleagues revealed that double knockout *Cry1* and *Cry2* leads to the ascended expression of gluconeogenic genes and enhanced hepatic glucose production [Zhang et al., 2010].

In addition, PER2 depletion disturbs glucocorticoid rhythmicity and protects mice from developing glucose intolerance in response to glucocorticoids [So et al., 2009]; [Yang et al., 2009].

2.5.2. Renal

Clock mutation is associated with renal sodium reabsorption. This means that Clock KO mice have lower arterial blood pressure than wild-type [Zuber et al., 2009]. Circadian rhythm also affects plasma renin, AngII and aldosterone levels [Kawasaki et al., 1990]. Similarly, mice with *Per1* mutations showed lower arterial blood pressure on a normal diet and a normal light/dark cycle [Stow et al., 2012].

2.5.3. Cardiovascular Diseases and Hypertension

Bray and colleagues showed that the overexpression of the *Clock*^{Al9} allele in cardiomyocytes changes heart rate instability and brings out a function of the peripheral circadian gene system in the control of cardiac fuel handling [Bray et al., 2008]. The *Bmal1* mutation also impaired circadian alterations in the blood pressure and heart rate, which leads to ascended sensitivity to vascular injury and skeletal muscle pathologies [Anea et al., 2009]; [Curtis et al., 2007]; [McCarthy et al., 2007]. Similarly, vascular impairment of *Pparγ*, which is a inducer of *Bmal1*, generates an important reduction in the daily fluctuation in heart rate and blood pressure [Berger, 2005]; [N. Wang et al., 2008]. Besides, genetic variants in BMAL1 are also associated with hypertension and type 2 diabetes, whereas single nucleotide polymorphism [SNP] in CRY2 has been linked with fasting glucose concentrations [Woon et al., 2007]; [Dupuis et al., 2010].

2.5.4. Lipid metabolism

When mice that lack the *Bmal1* repressor REV-ERB α were exposed to a shorter circadian cycle in constant darkness, they show altered lipid and bile metabolism [Preitner et al., 2002]; [Le Martelot et al., 2009]. Besides, Kornmann and colleagues indicated that the liver-specific overexpression of *Rev-erba* promotes alterations in expressions related with xenobiotic detoxification, carbohydrate metabolism, energy metabolism and lipid and sterol homeostasis genes [Kornmann et al., 2007].

2.5.5. Cancer

Our predecessors evolved to be diurnal; normally, they were active during day and rested at night under dark conditions. Thomas Edison's invention of the light bulb in 1879 has changed this situation; supported by artificial light, human lifestyles have changed significantly. Contrary to our diurnal nature, we can be active at night. Additionally, during periods of darkness in which our eyes would normally be exposed to only very low light intensity, they are often subjected to high light intensity. Light-at-night (LAN) sources can be many things, as shown in Figure 2.10 [Haim and Portnov, 2013].

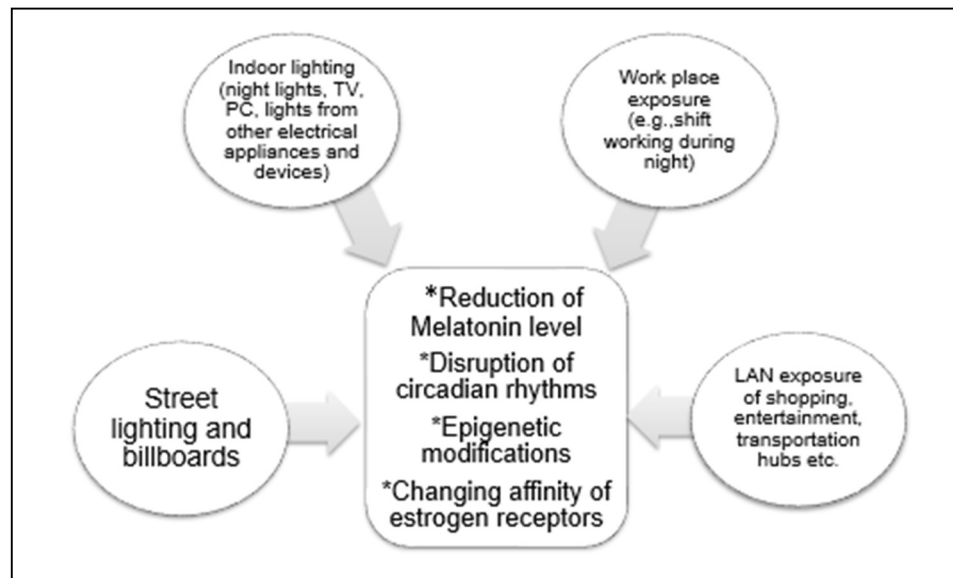


Figure 2.10: Relationship between LAN and cancer. LAN sources include street lighting, billboard lights, shopping centres and indoor lighting in our workplaces and homes. This light pollution leads to breast and prostate cancers.

Investigators define only ~25% of the workforce as employed in 'regular' daytime ($\pm 08:00$ to $\pm 17:00$ h) Monday-to-Friday work; shift work schedules disturb the natural sleep–wake cycle [Haus and Smolensky, 2013].

Melatonin functions both as a free-radical sweeper and has antioxidant properties through the stimulation of antioxidative enzymes [Tan et al., 2003]. Additionally, melatonin has been indicated as functioning in preventing tissue damage in aging and in an extensive range of disease states including cancer, dementia and stroke [Vijayalaxmi et al., 2002]; [Reiter, 2003]; [Reiter, 2004]; [Reiter et al., 2004];

[Reiter et al., 2005]. Disrupting the melatonin level also affects the immune system. Decreased melatonin level in those subjected to LAN can result in increased breast and prostate cancers risk via a greater proliferation of cancer cells and/or indirectly via immune system malfunction [Haim and Portnov, 2013].

Viswanattan and colleagues showed that women working night shifts for a long period have a significantly increased risk of endometrial cancer [Viswanattan, Hankinson, and Schernhammer, 2007]. Similarly, women who work a rotating night shift for 15 or more years may have increased risk of colorectal cancer [Schernhammer et al., 2003]. Related to this, women who have worked for least half a year in any trade with predominantly night work have a 1.5-fold higher risk of developing breast cancer [Hansen, 2001].

Graveyard shift working rotating night shift that exposure to light at night may ascend the risk of breast cancer [Davis, Mirick and Stevens, 2001]. The functional role of SCN with regard to tumour growth and the disruption of SCN accelerates malignant growth by 2–3-fold for two established tumours with different proliferation rates [Filipski et al., 2002]. It was demonstrated that the relationship between the intracellular circadian rhythm and the circadian clock controls the expression of cell cycle-related genes. With reference to the suppression of *Per1*, *Per2* and *Cry1* gene expression in breast cancer can cause the deregulation of cell cycle control [Matsuo et al., 2003]. The other survey results have shown that the disruption of *Cry* decreases cancer risk in *p53* mutant mice. These results provided that *Cry* mutation conserves *p53* mutant mice from the early onset of cancer [Ozturk et al., 2008].

2.6. Epigenetics, Circadian Rhythm Disruption and Cancer

The epigenetic regulation of DNA is significant for gene expression and altered gene transcription. DNA methylation is the most common aspect of epigenetic regulation. It occurs with the addition of a methyl (CH₃) group at the carbon 5 position in the cytosine [Das and Singal, 2004]. This cytosine is located within the CpG dinucleotide, known as CpG islands, and normal human DNA methylates 3–6% of all cytosines. CpG islands are usually unmethylated in all normal tissues [Mulero-Navarro and Esteller, 2008]. CpG islands are present on both housekeeping genes and genes

with a tissue-specific pattern. They are present in approximately half of all genes in humans [Das and Singal, 2004].

DNA methylation has an important role in the regulation of gene expression, differentiation and development in cancer. The methylation of cytosines in promotor-region CpG dinucleotides is a widespread event for the suppression of the tumour suppressor gene. This mechanism is a characteristic property of cancer cells. Many investigated genes have been shown to undergo hypermethylation in cancer [Shivapurkar and Gazdar, 2010].

Ras association domain-containing protein 1 (*RASSF1A*) and *p16* are widespread methylated in various cancers. In opposition to this, the *GSTP1* gene is hypermethylated in > 90% of prostate cancers when it is largely unmethylated in acute myeloid leukaemia. The methylation of the tumour-suppressor *RASSF1A* is increased in the benign breast epithelium of women. It is also associated with breast cancer risk [Haus and Smolensky, 2013]. The *BRCA1* gene is the most common gene that is used as a biomarker in breast cancer [Das and Singal, 2004]. *GSTP1* is a significantly good biomarker for prostate cancer because it is hypermethylated in 80–90% of cases. *p16INK4a* gene hypermethylation is associated with colorectal and lung cancer [Mulero-Navarro and Esteller, 2008]. However, the promoter methylation of *APC*, *RARβ2* and *RASSF1A* increase in benign breast cancer and they may be used as biomarkers [Lewis et al., 2005]. In addition, *MGMT* promoter hypermethylation may also present a functional biomarker for β-cell lymphomas [Esteller et al., 2002]. Moreover, *DAPK*, *p16*, *MGMT* and *IGFBP3* methylation have each been detected as having negative prognostic significance in early-stage non-small cell lung cancer [Issa, 2003]. Furthermore, the *APC* gene is a tumour suppressor gene and the methylation of the promoter 1A region correlates with non-small cell lung cancer tumours and breast cancer [a.K. et al., 2001].

Studies have revealed that the methylation of circadian genes is strongly associated with cancer. Specifically, many endocrine and metabolic hormones secretions are controlled by the day/night cycle; therefore, the aberrant regulations of biological clock genes are specifically associated with endocrine malignances. Hormone-related breast and prostate cancers are highly relevant to shift workers [Gery and Koeffler, 2010].

The promoter methylation of circadian genes happens often in breast cancer tissues [Kou S. et al., 2009]. It is known that promoter methylation silences the

expression of affected circadian genes and disrupts the circadian rhythm of breast cancer cells. The suppression of expression for the circadian clock's critical components PER1, PER2, BMAL1 and CRY1 may influence on the development of breast cancer. Circadian genes may function as oncogenes or tumour suppressors, because they are all related to cell proliferation, apoptosis, cell cycle control and the DNA damage response. However, the hypermethylation of *Clock* promoter decreased the risk of breast cancer [Hoffman et al., 2010]. The development of haematologic malignancies, non-Hodgkin lymphoma and acute lymphocytic leukaemia are conducted by the promoter methylation of *Bmal1* gene [Taniguchi et al., 2009]. In addition, the combination of low expression *Cry1* and *Bmal1* genes is an independent prognostic factor in epithelial ovarian cancer [Tokunaga, 2008]. It was found that the deregulation of *Per* genes most probably happens due to *Per1* and *Per2* promoter methylation and the expression of *Per1*, *Per2* and *Per3* genes is disturbed in > 95% of breast cancer in Taiwanese women. Moreover, the methylation of *Per* gene promoters is strongly correlated with *c-erbB2* expression [Chen S et al., 2005] and p53 regulation is under the circadian rhythm and regulates *Per2* gene expression [Miki et al., 2013]. Human breast and endometrial cancers demonstrated that the loss and deregulation of PER proteins is widespread in tumour cells [C. C. Lee, 2006]. Related to leukaemia, *hPER2* gene CpG sites were methylated in 40% of chronic myeloid leukaemia [CML], and the loss of *hPER3* transcription in CML is related to the methylation [Yang M. Et al., 2006].

2.7. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ Cas System

Many methods have improved genome editing, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the RNA-guided CRISPR-Cas nuclease system. Studies in recent years have shown that the CRISPR-Cas system will be commonly used in the future because the system is easier to design, efficient, more specific and uses multiplexed gene editing to ensure the diversity of cell types and organisms than other gene editing methods [Ran et al., 2013].

The CRISPR-Cas system consists of two major Cas enzymes and single guide RNA (sgRNA). Single guide RNA is a 20-nucleotide guide sequence that helps Cas9

find a target locus. The other molecule Cas9 is a nuclease and promotes double stranded break (DSB) at a target locus. Two DNA damage repair pathways can repair the DSB: the error-prone NHEJ and the high-fidelity HDR pathway. Both can be used to accomplish a demanded editing result [Perez et al., 2012].

If there is no repair template, DSBs are re-ligated via the NHEJ system. This process separates nicks in the insertion/deletion (indel) mutations. Indels comprised within an exon can cause frameshift mutations and premature stop codons. Therefore, NHEJ can be utilised to mediate gene editing. The other DNA repair pathway is the HDR system, which works in the repair template presence and ensures effective small gene edits in the genome [Chen et al., 2011].

Cas9 nucleases can carry an aspartate-to-alanine (D10A) mutation in the RuvC catalytic domain, this form is called a *Cas9 nickase mutant* that nicks rather than cleaves DNA to produce single-stranded break. Offset sgRNA pairs can guide the Cas9 nickase mutant and increase the target locus' specificity. Wild-type Cas9 nuclease or mutant Cas9 nickase can ease eukaryotic genome editing [Ran et al., 2013]. Figure 2.11 shows both the wild-type Cas9 and the Cas9 nickase mutant mechanisms.

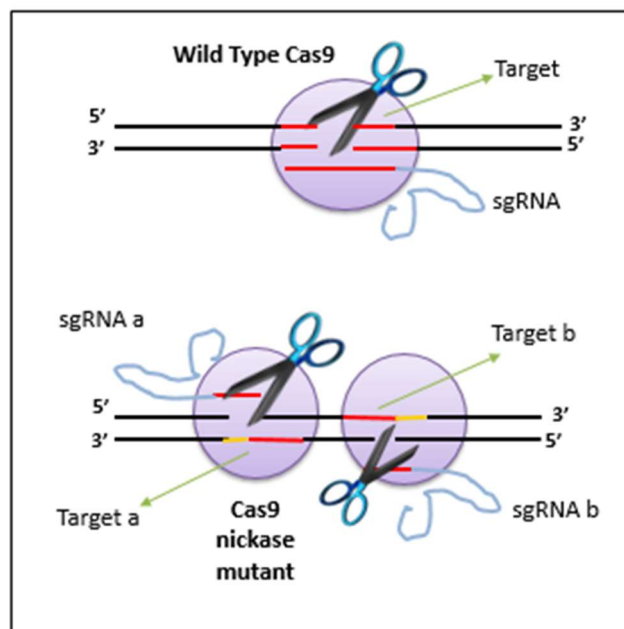


Figure 2.11: Wild-type Cas9 and Cas9 nickase mutant nicking mechanism.

2.8. High Resolution Melting (HRM) Analysis

Several methods have been developed to detect DNA methylation, such as HPLC, ELISA-based, RFLP, and Pyrosequencing. One, High Resolution Melting (HRM) analysis is used for methylation profile detection [Kurdyukov and Bullock, 2016]. The methylation analysis experimental workflow is as shown in Figure 2.12.

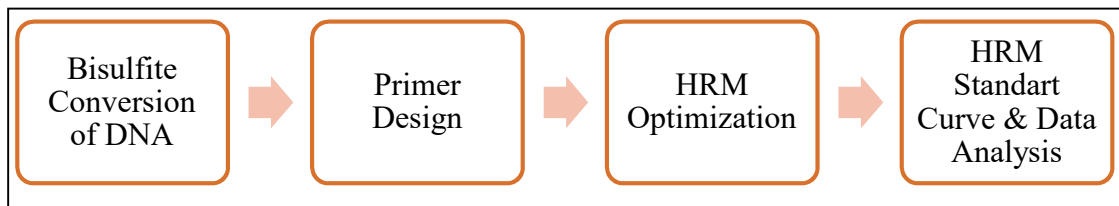


Figure 2.12: Methylation analysis experimental workflow

In this method, DNA is first treated with bisulfite. In this based, unmethylated cytosines are converted to uracil and recognised as thymine following PCR amplification. However, methylated cytosines do not convert to uracil and remain as cytosine. Therefore, methylated cytosines are distinguished from unmethylated cytosines (Figure 2.13) [Li and Tollefsbol, 2011]. Methylated DNA and unmethylated DNA obtain different sequences. The resulting PCR products have markedly different melting profiles (Figure 2.14) [Wojdacz and Dobrovic, 2007].

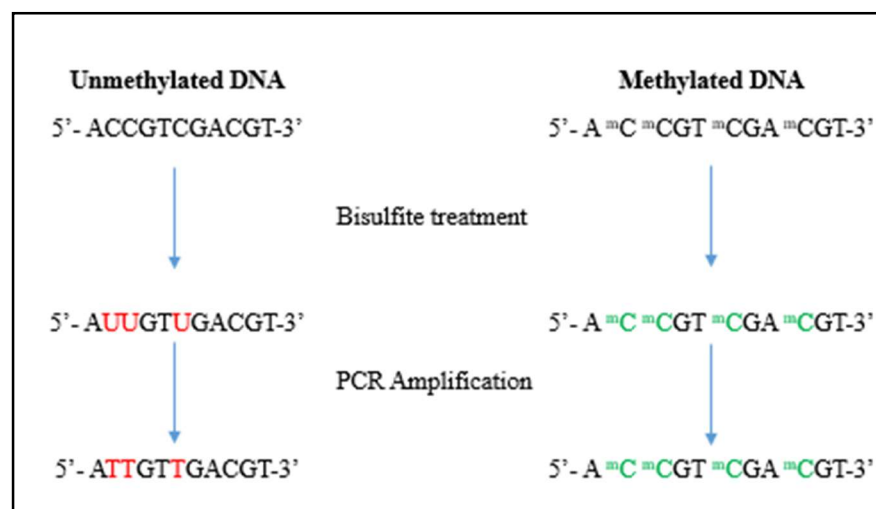


Figure 2.13: Bisulfite conversion of DNA

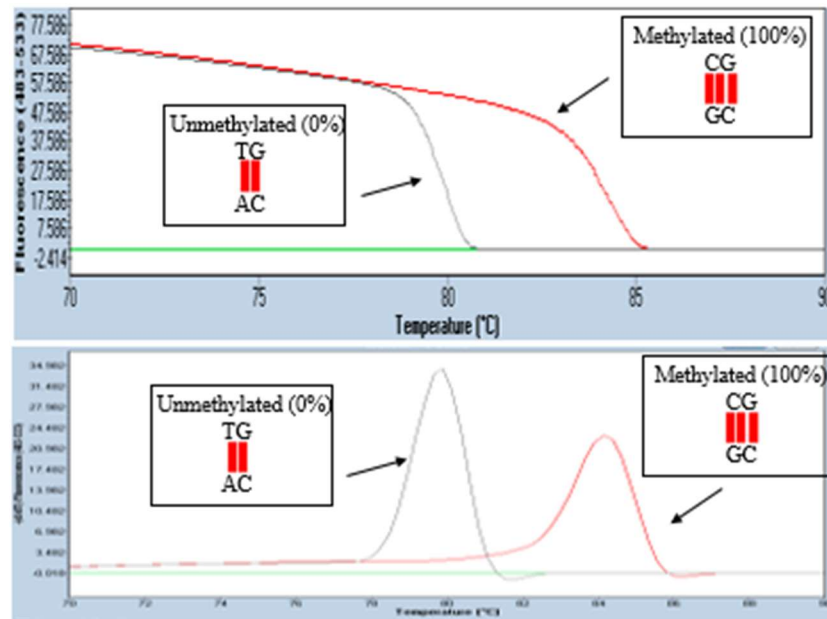


Figure 2.14: Differences between PCR products were distinguished by melting temperature (T_m)

The advantages of HRM analysis are that it is cost effective, offers a simple and fast workflow and has a low reagent consumption compared to alternate methods. Performing HRM analysis requires saturating fluorescent dyes that are used to monitor the denaturation of sample DNA amplicons. HRM is optimised by GC-rich targets (GC% ~65–72.5) [Wittwer, 2009].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. General Kits and Reagents

- Cell Culture: MCF10A (Cat. No. CRL-1031) and MDA-MB-231 (Cat. No. HTB-26) cell lines were supplied by American Type Culture Collection (ATCC).
- Genome Editing: The Stbl3 strain derived from the E. coli bacterium was used in cloning protocols. pX462 (#48141), and LentiCRISPR.V2 (#52961) were obtained from Addgene.

The reagents and kits used in cell culturing and genome editing are as shown in Table 3.1.

Table 3.1: General kits and reagents used in cell culture and genome editing

Reagent	Manufacturer
MEGM™ Mammary Epithelial Cell Growth Medium	MEGM BulletKit Cat No: CC-3150, Lonza, Switzerland
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific
Cholera toxin	Sigma
Non-essential amino acids	Thermo Fisher Scientific
Penicillin / streptomycin	Thermo Fisher Scientific
Epidermal growth factor (EGF)	Sigma
Insulin	Sigma
Hydrocortisone	Sigma
Dulbecco's Modified Eagle Medium (DMEM)	41965-039, Gibco Invitrogen, USA
MNagel midiprep Kit	Macherey Nagel, Germany
MNagel gel isolation Kit	Macherey Nagel, Germany

- **Western Blot:** 1X Lysis Buffer and 1X PBS were used to prepare samples. 1X SDS Running Buffer was used in the SDS-PAGE Gel Electrophoresis step. 1X Transfer Buffer was used to transfer the protein from the gel to the membrane. The membrane was taken into a case filled with 5% blocking solution and 1X TBST was used for the membrane wash. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation (Western Blotting Detection Kit Advansta Cat: K-12043-D10 and Signal Fire™ ECL Reagent, Cell Signalling Cat: 6883S substrates were used. Western Blotting Detection Kit Advansta includes Western Bright Peroxide (Component 1) and Western Bright Sirius (Component 2)).
- **DNA Extraction:** Buffer and solution for DNA isolation were obtained by NucleoSpin® Tissue Kit (Macherey-Nagel).
- **Bisulfite Conservation:** Buffer and solution for Bisulfite Conservation were obtained using a EZ DNA Methylation-Lightning™ Kit (Zymo Research) (Cat. Nos. D5030 and D5031).
- **PCR:** We used different high-resolution melting dyes to decide which gave the best result. A Human Methylated and Non-methylated DNA Set was used as a control genomic DNA (Zymo Research, Cat. No D5014) and Bisulfite-Converted Universal Methylated Human DNA Standard (Zymo Research, Cat. No. D5015) was studied to check the PCR efficiency. The commercial kits used were as shown in Table 3.2.

Table 3.2: Commercial kits used for optimization

Kit Name	Catalog No	Brand
LightCycler 480 High Resolution Melting Master	4909631001	Roche
MeltDoctor HRM Master Mix	4415440	ThermoFisher
ZymoTaq™ qPCR Premix	E2054	Zymo Research
SensiFAST SYBR	BIO-98005	Bioline

- **Gel Electrophoresis:** Agarose gel electrophoresis was performed to visualize genomic DNA and PCR products. The method's buffers and solutions were as given in Table 3.3.

3.1.2. Buffers and Solutions

Table 3.3: List of buffer and solutions

5 x TBE buffer	54 g Tris 27.50 g Boric acid 20 ml 0,5 M EDTA
10 x loading buffer	2.5 mg/ml bromophenol blue 1% SDS in 2 ml of glycerol
Ethidium Bromide	10 mg/ml (Thermo Fisher)
Agarose	BioShop® Canada Inc Burlington
1 x TE	10 mM Tris.Cl 1 mM EDTA pH 8.0
Alcohol	Merck, Germany
Primer-probe dilution buffer	10 µM Tris 0,1 µM EDTA
GeneRuler λ Hind III Marker	SM0103, Fermentas
GeneRuler 100 bp DNA Ladder	SM024, Fermentas
LB Broth-Lennox	Sigma
NEB 2 buffer	50 mM NaCl 10 mM Tris-HCl 10 mM MgCl ₂ 1 mM DTT
T4 Polinükleotid kinaz	ThermoFisher Scientific
T4 Ligaz	ThermoFisher Scientific
1X Lysis Buffer	1X Laemmli Buffer 1% Beta-Mercaptoethanol
10X PBS	BE17-517Q, Lonza, Belgium
1X SDS Running Buffer	250 Mm Tris-Base (pH: 8.3) 11.90 M Glycine 1% SDS
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol

Table 3.3: Continued

5% Blocking Solution	5% non-fat dry milk in 11X TBS-T
1X TBST	1X TBS 0.1% Tween-20
10X TBS	200 Mm Tris-Cl (pH: 7.6) 1.5 M NaCl
4X Protein Loading Dye (Laemmli)	250 Mm Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 20% β -mercaptoethanol 0.008% Bromophenol Blue
Cell Lysis Buffer (RIPA)	750 mM NaCl 250 mM Tris (pH: 8.0) 0.1% SDS 1.0% NP-40
Dulbecco's Modified Eagle's Medium - low glucose	D2902, Sigma-Aldrich, USA

3.1.3. Equipment

Table 3.4 lists the machines and equipment used in this study.

Table 3.4: List of equipment

RT-qPCR system	Roche LightCycler 480 II Real Time PCR System
PCR Thermal Cycler	Applied Biosystems Veriti 96-Well Thermal Cycler
Trans-Blot Turbo Transfer System	Biorad Transblot Turbo Transfer System, Serial No: 690BR011760

Table 3.4: Continued

Chemiluminescent Signals Imager	BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545
Inverted Microscope	Nikon, Eclipse, E100
Incubator (CO ₂)	Thermo-Scientific, USA
Agarose Gel Electrophoresis	VWR® Midi Plus Horizontal Electrophoresis Systems
Laminar Flow Cabinet	Hera-Safe Class II Cabinet, Thermo Scientific, USA
NanoDrop	ND-1000 Spectrophotometer V3.5
UV-Transilluminator	VWR® UV Transilluminators
Centrifuge	Sigma 1-14 Microfuge
Software	LightCycler 480 Software release 1.5.0
Water Purification System	Elga PURELAB Flex 3 Water Purification System
Vortex	VWR® Vortex Mixers
Shaker	MSC-100 Thermo Shaker Incubator/ ALLSHENG
Printer	Sony Digital Graphic Printer UP-D897
Pipets	Gilson, Inc PIPETMAN Pipettes
Tips	Neptune® BT Barrier Pipet Tips

3.1.4. Oligonucleoside Primers

Table 3.5 gives detailed information about oligonucleotide primers.

Table 3.5: Used oligonucleotide primers

Primers	Primer Sequences (5'-3')	Target gene	T _m (°C)	Amplicon size (bp)	Synthesized by
BRCA1-F	GGAAAAGCGCGGG AATTATAG	Breast Cancer susceptibility gene 1	60.4	79	Sentromer
BRCA1-R	ATCCCCCGTCCAA AAAATCTCAAC		61.0		
RASSF1A-F	GGGACTTTGAGTT TATTGAGTTG	Ras-association domain family 1 isoform A	57.1	152	Genotech
RASSF1A-R	CCACAACCAAAAA CCAACCTACC		58.4		
RAR β -F	AGTTGTTTGAGGA TTGGGAT	Retinoic acid receptor beta	53.2	183	Genotech
RAR β -R	TCTCCTTCCAAATA AATACTTACA		54.2		

3.2. Methods

3.2.1. Cell Culture

i) Properties and Maintenance of Cell Lines

This study used two mammary epithelial cell lines, MCF10A and MDA-MB-231 to create a mutant platform of biological clock genes. The cells were maintained in a humidified incubator at 37°C under 5% CO₂.

The MCF10A (a nontumorigenic cell line in immune system deprived mice) is a mammary epithelial cell line obtained from a 36-year-old woman. This cell line grows as a monolayer and can be differentiated in an appropriate culture medium to become similar to cells in breast tissue.

First, MCF10A was grown in MEGM™ Mammary Epithelial Cell Growth Medium to create a stockpile. Genomic editing and subsequent steps were performed in DMEM supplemented with 10% FBS, 100 ng/ml cholera toxin, 1% non-essential amino acids, 1% penicillin/streptomycin, 20 ng/ml epidermal growth factor (EGF), 0.01 mg/ml insulin and 500 ng/ml hydrocortisone.

The other cell line used in this study was MDA-MB-231, which is an aneuploid cell line with invasive properties. This cell line was grown in DMEM medium that contained 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin.

3.2.2. Genome Editing

MCF10A and MDA-MB-231 cells were edited using the CRISPR technique in Prof. Dr. Aziz Sancar Biological Clock Laboratory at Gebze Technical University. CRISPR design [Ran et al., 2013] and CRISPR Design Tool [Hsu et al, 2013] were used for sgRNA design programs to select efficient sgRNA for the *Clock*, *Bmal1* and *CRY 1and2* genes.

In this thesis, mutations were made using wild-type cas9 (from LentiCRISPR v2) and nickase mutant cas9 (form pX462).

3.2.2.1. Cas9 Nickase Mutant-mediated Disruption of Circadian Clock Genes

The Stbl3 strain derived from *E. coli* was used in the cloning protocols for non-stable vectors. A cloning study of pX462 plasmid was performed with Stbl3 and the oligomers used with nickase Cas9 were given in Table 3.6.

Table 3.6: Oligomers used with Nickase Cas9. Oligos were used with Nickase-type Cas9 (pX462). Two genes (P1 and P2) were genetically engineered in the genomes close to each gene to encode the gene coding sequence and these were tested in studies to see whether the phenotypes obtained with P1 and P2 supported one of them. For each P target, the left (L) or right (R) oligo pairs close to each other were used in the genome. P: Target selected for Nickase. L: Left area of the target, R: Right cut area of the target

24 bp	CRY1-P1-L-S	CACCGTAGACGCAGCGGATGGTGT
24 bp	CRY1-P1-L-A	AAACACACCATCCGCTGCGTCTAC
25 bp	CRY1-P1-R-S	CACCGTTCGCCGGCTCCTCCAATGT
25 bp	CRY1-P1-R-A	AAACACATTGGAGGAGCCGGCGAAC
25 bp	CRY2-P1-L-S	CACCGCCAGCAACGCCGGGTGTCG
25 bp	CRY2-P1-L-A	AAACCGACAACCCGGCGTTGCTGGC
24 bp	CRY2-P1-R-S	CACCGCGTTTACATTCTCGACCCG
24 bp	CRY2-P1-R-A	AAACCGGGTCGAGAATGTAAACGC
25 bp	Bmal-P1-L-S	CACCGACCCTGATTTCCCGTTCAC
25 bp	Bmal-P1-L-A	AAACGTGAACGGGGAAATCAGGGTC
24 bp	Bmal-P1-R-S	CACCGTTTCTCGGCACGCGATAGA
24 bp	Bmal-P1-R-A	AAACTCTATCGCGTGCCGAGAAAC
25 bp	Clock-P1-L-S	CACCGACCTCTGAATGTTCCCTTC
25 bp	Clock-P1-L-A	AAACGAAGGGGAACATTCAGAGGTC
25 bp	Clock-P1-R-S	CACCGAATACTCTCTACTCATCTGC
25 bp	Clock-P1-R-A	AAACGCAGATGAGTAGAGAGTATTC

3.2.2.2. Cas9 Wild-type (Lentivirus)-mediated Disruption of Circadian Clock Genes

i) Amplification of plasmids

- 5 ng of plasmid DNA was added to 50 µl of competent cells. Samples were kept on ice for 30 minutes.
- A heat shield was applied at 42°C for 60 seconds. The samples were placed in a 1.5 ml centrifuge tube and put on ice for two minutes.

- After 900 µl of LB Broth-Lennox medium was added to the samples, they were incubated at 37°C on a shaker that was rotating at 200 rpm.
- Following a one-hour transformation, bacteria including plasmids were spread on LB-Agar platelets containing 100 µl of the sample ampicillin. It was incubated at 37°C.
- Single-cell derived colonies were transferred to 5 ml of LB broth containing ampicillin and grown for 20 hours.
- The bacteria were settled at 4,500 rpm for five minutes to obtain plasmid-containing bacterial pellets.
- Plasmids were isolated with a MNagel midiprep kit and dissolved in 1 x TE buffer. Stored at -20°C.
- Plasmids were verified by cutting with appropriate enzymes. The pX459 v1 (version 1) and orv2 (version 2) plasmids were digested with Bbs I restriction enzyme and LentiCRISPR.V2 plasmid was digested with BsmBI restriction enzyme for 2-4 hours.
- The plasmids were ligated to the agarose gel with a MNagel gel isolation kit and washed. The samples were eluted to double distilled water.
- The oligos corresponding to sgRNA in the CRISPR system listed in Tables 3.6 and 3.7 were obtained commercially.

ii) Cloning Procedure

- 50 ng/ml double stranded oligos were left at 98°C in NEB 2 (New England Biolabs) buffer for five minutes. The heater was closed and allowed to hybridise until it reached 25°C.
- 50 ng of the digested plasmid and 150 ng of the hybridization oligos were added to a final volume of 10 µl in a final volume of 1 x T4 ligase buffer. Afterwards, 0.5 µl T4 Polinükleotid kinase and 1 µl T4 Ligase were added, it was left for 15 minutes at room temperature to allow a phosphorylation reaction. It was then incubated overnight at 4–8°C for the ligation reaction.
- 5 µl of the ligation products were transferred to 100 µl competent E. coli Stbl3 bacteria for the heat shock method.

- After bacteria were transferred to 900 µl LB, they were incubated at 37°C for one hour.
- Bacteria were sown on the LB-agar medium containing ampicillin (100 µg/ml) and incubated at 37°C for 16–18 hours to grow plasmid-containing colonies.
- At the end of the cloning process, bacteria were transferred to a liquid medium with 3 ml of LB + ampicillin (100 µg/ml) and grown at 37°C for 18–20 hours.
- Bacterial culture was precipitated at maximum speed (14,000 rpm) with the aid of a micro-centrifuge.
- After the supernatant was removed with the assistance of a vacuum, candidate constructs were isolated according to the protocol of the MNagel miniprep kit isolated from three columns for each candidate gene.
- Candidates were selected with appropriate restriction enzyme combinations and inserts were selected according to fragment length analysis.
- The samples obtained from the two columns for each gene were confirmed by sequencing.
- pX459- and p462-based constructs were prepared for use in cell culture transfection studies by isolating them with the midiprep kit as described in the propagation of the plasmids given above.

Table 3.7: Oligomers were used with wild-type Cas9 (pX459, Lent, CRISPR.v2). Two different (T1 and T2) loci were targeted in the genome close to each gene encoding the gene coding sequence and tested in some studies to support one of the T1 and T2 phenotypes. T: Target

25 bp	CLOCK-T1 Sen	CACCgAAATGAGCTCGATTGTTGAC
25 bp	CLOCK-T1Ant	AAACGTCAACAATCGAGCTCATTTc
25 bp	CLOCK-T2 Sen	CACCgTCTAGCATTACCAGGAAGCA
25 bp	CLOCK-T2 Ant	AAACTGCTTCCTGGTAATGCTAGAc
25 bp	BMAL1-T1 Sen	CACCgTGTTCTGTATATTCTAACCT
25 bp	BMAL1-T1 Ant	AAACAGGTTAGAATATACAGAACA c
25 bp	BMAL1-T2 Sen	CACCgTAGATAAACTTACTGTGCTA
25 bp	BMAL1-T2 Ant	AAACTAGCACAGTAAGTTTATCTAc
25 bp	CRY1-T1 Sen	CACCgTGAATGCACTCCTTCAGGGC
25 bp	CRY1-T1 Ant	AAACGCCCTGAAGGAGTGCATTCAc
25 bp	CRY1-T2 Sen	CACCgCCTTCAGGGCGGGGTTGTCG
25 bp	CRY1-T2 Ant	AAACCGACAACCCCGCCCTGAAGGc
25 bp	CRY2-T1 Sen	CACCgACAGCGCCTCTTCGGTGCAC
25 bp	CRY2- T1 Ant	AAACGTGCACCGAAGAGGCGCTGTc
24 bp	CRY2-T2 Sen	CACCGCTGCGACTCCACGACAACC
24 bp	CRY2-T2 Ant	AAACGGTTGTCGTGGAGTCGCAGC

ii) Preparing Lentiviral particles, Transfection of pX462 system (Cas9 Nickase mutant) and Wild-type Cas 9 (Lentivirus) Infection:

- Lentiviral particles were prepared by co-transfecting LentiCRISPRV2 with target sequences.
- After 48 hours of transfection, media including lentiviral particles were collected and filtered with a 0.2 mm filter; it was kept at -80°C.
- MCF10A and MDA-MB-231 cells were infected with lentiviral particles in 12-well plates in 1 ml of total medium (0.5 ml fresh culture medium and 0.5 ml medium containing viral particles) that contain 8 ng/ml Polybrene (Santa Cruz).
- For the pX462 system (Nickase Cas 9), two vectors encoding for sgRNAs of target sites were co-transfected into MDA-MB-231 cells using PEI as a transfection agent as explained in the previous section (Ehrhardt et al., 2006).
- Infected and transfected cells were selected with Puromycin (0.5 mg/ml for MCF10 and 1.5 mg/ml for MDA-MB-231) for transfected or non-infected cells

were killed over four days. Then, single cell-derived clones were picked-up by serial dilution.

- Candidates were first raised by transferring them to 24-well plates. They were then transferred to 12-well platelets. Subsequently, candidates were grown in six-well plates in duplicates. They were rapidly digested with 1x Laemm Buffer solution and screened with immunoblotting analysis to select knockout ones.
- Then, at least two cell lines were detached with RIPA for each knockout produced from different monoclonal while being transferred to T25 cell flasks.
- It was confirmed by immunoblot control. Then frozen in liquid nitrogen in a DMEM culture medium containing 10% DMSO and fed with 20% FBS.

We had planned to obtain knockouts with Lentivirus and the pX462 system (as in MDA-MB-231). For MCF10A cells, we had difficulty with the pX462 system, so we also targeted another site (T2) with the Lentivirus.

3.2.3. Western Blot Analysis

3.2.3.1. Sample Preparation

Cells were lysed directly in 1X lysis buffer that contains 1X Laemmli Buffer with added 1% Beta-Mercaptoethanol. After the medium was removed, the cells attached to the monolayer were washed with 1X PBS, and then 1X lysis buffer was added on to wells according to the cell confluency. After incubation for 10 minutes at room temperature with shaking, cells were transferred from the plate into Eppendorf tubes.

3.2.3.2. SDS-PAGE Gel Electrophoresis

Samples were incubated at 95°C for 10 minutes, and then they were briefly spun. Samples were loaded each well of the gel respectively and 1X SDS Running Buffer was used for electrophoresis. The gel was run at 80 V until proteins reached the separating gel, then the voltage might be increased up to 120–150 V.

3.2.3.3. Transferring the Protein from the Gel to the Membrane

The Trans-blot Turbo Transfer System (Biorad Transblot Turbo Transfer System, Serial No: 690BR011760) was used for the transfer. Four blotting papers and one nitrocellulose membrane with the same dimensions as the SDS PAGE gel were cut. The blotting paper and nitrocellulose membrane were incubated with 1X transfer buffer and a transfer sandwich was then created.

Assembling the Blotting Sandwich: The components were assembled on the cassette in the following order: Top (-) Cassette electrode (cathode)–Two Blotting papers–Gel–Blotting Membrane–Two Blotting papers–Bottom (+) cassette electrode (Anode). A roller was used to remove any air trapped between the layers. Transfer occurred at 12 V constant voltage for 90 min at the Transblot transfer system.

3.2.3.4. Antibody Incubation

The membrane was taken into a case filled with 5% blocking solution for one hour at room temperature after the transfer had completed. The primary antibody was diluted in 5% blocking solution as recommended in its datasheet and the membrane was incubated with it overnight at 4°C. The membrane was then washed four times (for 5, 15, 5, and 5 minutes, respectively) with 1X TBST, and was then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in TBST as recommended in its datasheet) for one hour at room temperature.

3.2.3.5. Chemiluminescence Imaging of the Membrane

At the end of the secondary antibody incubation, the membrane was washed four times with TBS-T. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation (In Prof. Dr. Aziz Sancar Biological Clock Laboratory, Western Blotting Detection Kit Advansta Cat: K-12043-D10 and Signal Fire TM ECL Reagent, Cell Signalling Cat: 6883S substrates are used. The Western Blotting Detection Kit Advansta includes Western Bright Peroxide (Component 1) and Western Bright Sirius (Component 2). The Working solution mix is prepared with an equal aliquot of components 1 and 2. The membrane is exposed to

the mix for two minutes (Signal Fire). The chemiluminescent signals were captured using an imager (BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545).

3.2.4. DNA Extraction, Quality and Quantity of DNA

i) DNA Extraction

DNA was extracted from 10^6 cells using the NucleoSpin® Tissue Protocol provided with the NucleoSpin® Tissue Kit (Macherey-Nagel).

- 180 µl Buffer T1 and 25 µl Proteinase K solution were added and vortexed.
- The mixture was incubated at 56°C in a water bath until the tissue was completely lysed (1–3 hours). During incubation, the mixture was vortexed for 15 s.
- 200 µl Buffer B3 was added to sample and vortexed vigorously. The sample was incubated at 70°C for 10 min and vortexed briefly.
- 210 µl ethanol (96–100%) was added to the mixture and this was vortexed to yield a homogenous solution.
- The homogenous solution was pipetted into the NucleoSpin® Tissue Column in a 2 ml collection tube.
- The homogenous solution was centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the column was returned into the collection tube.
- 500 µl of Buffer BW was added and centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the column was returned into the collection tube.
- 600 µl of Buffer B5 was added to the column and centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the column was returned into the collection tube.
- The column was centrifuged for 1 min at 11,000 x g and the collection tube was removed.
- The NucleoSpin® Tissue Column was placed in a clean 1.5 ml microcentrifuge tube and 100 µl buffer BE (70°C) was pipetted directly on to the NucleoSpin® membrane and was incubated at room temperature for 1 min.

- This was then centrifuged at 11,000 x g for 1 min to elute.
- After the amount of DNA was measured by spectrophotometer, the DNA solution was stored at -20°C until it was used in processes of PCR.

Following DNA extraction, DNAs were run on a 0.8% agarose gel.

ii) Quantification of Nucleic Acids

The DNA concentration was determined using a spectrophotometer. Samples were exposed to 260 nm and 280 nm ultraviolet light. The ratio of 260/280 was in the range 1.6–2.0, which was indicative of pureDNA, while a low ratio was indicative of protein contamination [Sambrook and Russell, 2001].

3.2.5. Bisulfite Conversion

The conversion of non-methylated cytosines to uracil [Frommer et al., 1992], which left methylated cytosines unaltered, was achieved using the EZ DNA Methylation-Lightning™ Kit (Zymo Research). DNA was treated from 200–500 ng using the EZ DNA Methylation-Lightning™ Protocol.

- 130 µl Lightning Conversion Reagent were added to 20 µl DNA in PCR tube and mixed.
- The mixture was placed in a thermal cycler and the following steps were performed.
 - 98°C for eight minutes
 - 54°C for 60 minutes
 - 4°C storage up to 20 hours (optional)
- 600 µl M-Binding Buffer to Zymo-Spin™ IC Column was added and the column was placed into a provided Collection Tube.
- The sample (from Step 2) was added into a Zymo-Spin™ IC Column that contained the M-Binding Buffer. The cap was closed and mixed by inverting it several times.

- The homogenous solution was centrifuged for 30 seconds at full speed (16,162 x g). The flow-through was discarded and the column was returned to the collection tube.
- 100 µl of M-Wash Buffer was added and centrifuged for 30 seconds at full speed. The flow-through was discarded and the column was returned to the collection tube.
- 200 µl L-Desulphonation Buffer was added to the column and incubated for 15–20 minutes at room temperature (20–30°C). After incubation, it was centrifuged for 30 seconds at full speed.
- 200 µl of M-Wash Buffer was added and centrifuged for 30 seconds at full speed. This wash step was repeated.
- The Zymo-Spin™ IC Column was placed into a clean 1.5 ml microcentrifuge tube and 10 µl of M-Elution Buffer was directly added to the column matrix.
- This was centrifuged for 30 seconds at full speed.
- After the amount of bisulfite converted DNA was measured by a spectrophotometer, the converted DNA solution was stored at -20°C until it was used in PCR processes.

3.2.6. Primer Design and Production

We used BRCA1 primers from the study by Wojdacz and colleagues [Wojdacz et al., 2011]. We designed BRCA1 methylated and unmethylated probes by considering location, melting temperature, annealing temperature and GC content. BRCA1 probes were checked using OligoAnalyzer software. RASSF1A and RARβ primers and probes were designed and optimised at the TUBITAK Project No 214S321, Report 3 [Akyurek et al., 2018]. BRCA1 primers and probes were synthesised by Sentromer, Istanbul, Turkey; RASSF1 and RARβ primers and probes were synthesised by Genotech, South Korea.

Primer details are given in Appendices A, B and C.

3.2.7. High Resolution Melting (HRM) Dye Optimization

Different master mixes (as shown in Table 3.2) were tried using a Human Methylated and Non-methylated DNA Set (Zymo Research). The used reaction setups are as shown in Tables 3.8 and 3.9.

The LC 480 HRM Master Kit does not contain MgCl_2 . The MgCl_2 concentration was optimised using TUBITAK Project No 214S321, Report 2, and the best found concentration was 3 mM (Table 3.9) [Akyurek et al., 2017].

Table 3.8: Used reaction setups for ZymoTaq™ qPCR Premix, SensiFAST SYBR, MeltDoctor HRM Master Mix

Reagent	Volume	Final conc.
2X Master Mix	10 μl	1 X
Forward Primer (10 μM)	1 μl	1 μM
Reverse Primer (10 μM)	1 μl	1 μM
Template DNA	3 μl	100 ng/20 μl
ddH ₂ O	5 μl	-
Total Volume	20 μl	

Table 3.9: Used reaction setups for LightCycler 480 High Resolution Melting Master

Reagent	Volume	Final conc.
2X Master Mix	10 μl	1 X
Forward Primer (10 μM)	1 μl	1 μM
Reverse Primer (10 μM)	1 μl	1 μM
Template DNA	3 μl	100 ng/20 μl
MgCl_2	3 μl	3 mM
ddH ₂ O	2 μl	-
Total Volume	20 μl	

The used conditions demonstrated for qPCR were as listed in Table 3.10.

Table 3.10: Used conditions setups for qPCR

Initial denaturation	95 °C 10 min	1 cycles
Denaturation	95 °C 10 sec	42 cycles
Annealing	60 °C 15 sec	
Extension	72 °C 20 sec	
High Resolution Melting	95 °C 1 min	1 cycles
	40 °C 1 min	
	65 °C 1 sec	
Cooling	40 °C 10 sec	∞

ZymoTaq™ qPCR Premix were chosen and used during the HRM study. Analysis was performed using LightCycler® 480 Software, Version 1.5 (Roche).

3.2.8. Real-Time PCR (qPCR)

Amplification of BRCA1, RASSF1A and RARβ was performed with specific primers (as shown in Table 3.5) for each locus using the ZymoTaq™ qPCR Premix for Real-Time PCR. The master mix was a 2X concentrated ready-to-use mixture that contained optimised buffer, ZymoTaq qPCR. For each reaction tube 10µl (1x) ZymoTaq™ qPCR Premix, 1 µl of each forward and reverse primers from 10 µM 3 µl of 5 ng/µl template DNA samples and finally 5 µl ddH2O were added to reach a total reaction volume of 20 µl, as recommended by the manufacturer (Zymo Research). The amplification of BRCA1 was performed using in the Table 3.11 cycling parameters:

Table 3.11: Used conditions setups for BRCA1 qPCR

Initial denaturation	95 °C 10 min	1 cycles
Denaturation	95 °C 10 sec	45cycles
Annealing	57 °C 15 sec	
Extension	72 °C 20 sec	
High Resolution Melting	95 °C 1 min	1 cycles
	40 °C 1 min	
	65 °C 1 sec	
Cooling	40 °C 10 sec	∞

First, RASSF1A amplicons were performed for 45 cycles, but the negative control signed peak was reached after 42 cycles. Non-specific binds were observed. We tried 36, 40 and 42 cycles and found the best results for 42 cycles. Thus, 42 cycles were used as quantification. The amplification of RASSF1A was performed using in the Table 3.12 cycling parameters:

Table 3.12: Used conditions setups for RASSF1A qPCR

Initial denaturation	95 °C 10 min	1 cycles
Denaturation	95 °C 10 sec	42 cycles
Annealing	60 °C 15 sec	
Extension	72 °C 20 sec	
High Resolution Melting	95 °C 1 min	1 cycles
	40 °C 1 min	
	65 °C 1 sec	
Cooling	40 °C 10 sec	∞

Similarly, RAR β amplicons were performed for 42 cycles, but all DNAs showed peaks after cycle of quantification (Cq) 33. We decided to use 37 cycles for RAR β . The amplification of RAR β was performed using in the Table 3.13 cycling parameters:

Table 3.13: Used conditions setups for RAR β qPCR

Initial denaturation	95 °C 10 min	1 cycles
Denaturation	95 °C 10 sec	37 cycles
Annealing	58 °C 15 sec	
Extension	72 °C 20 sec	
High Resolution Melting	95 °C 1 min	1 cycles
	40 °C 1 min	
	65 °C 1 sec	
Cooling	40 °C 10 sec	∞

RASSF1A and RAR β annealing temperature optimizations were done as per Akyurek et al., 2018.

The following Real-Time PCR products were run on 2% agarose gel.

3.2.9. High Resolution Melting (HRM) Analysis

The PCR products generated with the ZymoTaq™ qPCR Premix for Real-Time PCR were subjected to HRM right after the reaction with the same instrument LightCycler 480 II (Roche Diagnostic) Real-Time PCR Detection System. HRM data were analysed using LightCycler 480 Software release 1.5.0 HRM analysis, which provided qualitative data.

4. RESULTS

4.1. Western Blot Analysis of Knock Outs of Clock, Bmal1 and Cry1:Cry2 Genes at the Protein Level for MCF10A Cells

4.1.1. Clock Gene Screening

The Western Blot screening of Clock knockout cell lines were as shown in Figure 4.1. According to the results shown at Figure 4.1, Clock gene is knockout when compared to wild type.

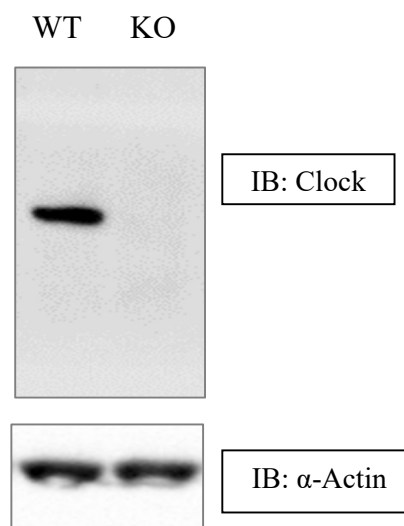


Figure 4.1: Western Blot analysis of transfected MCF10A cell extracts, Clock at 8% SDS-PAGE gel. WT: Wild type cell line; KO: Knockout cell line; IB: Immunoblot.

4.1.2. Bmal1 Gene Screening

The Western Blot screening of Bmal1 knockout cell lines were as shown in Figure 4.2. According to the results shown at Figure 4.2, *Bmal1* gene is knockout when compared to wild type.

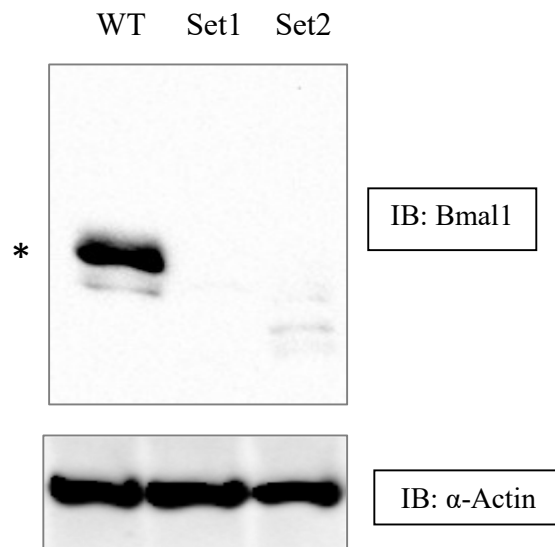


Figure 4.2: Western Blot analysis of transfected MCF10A cell extracts, Bmal1 at 8% SDS-PAGE gel. WT: Wild type cell line; Set1: Knockout 1; Set2: Knockout 2; IB: Immunoblot.

4.1.3. Cry1: Cry2 Gene Screening

Western Blot screening of Cry1:Cry2 knockout cell lines were as shown in Figure 4.3. According to Figure 4.3, *Cry1* and *Cry2* respectively, are knockouts when compared to wild type (both left and right figures).

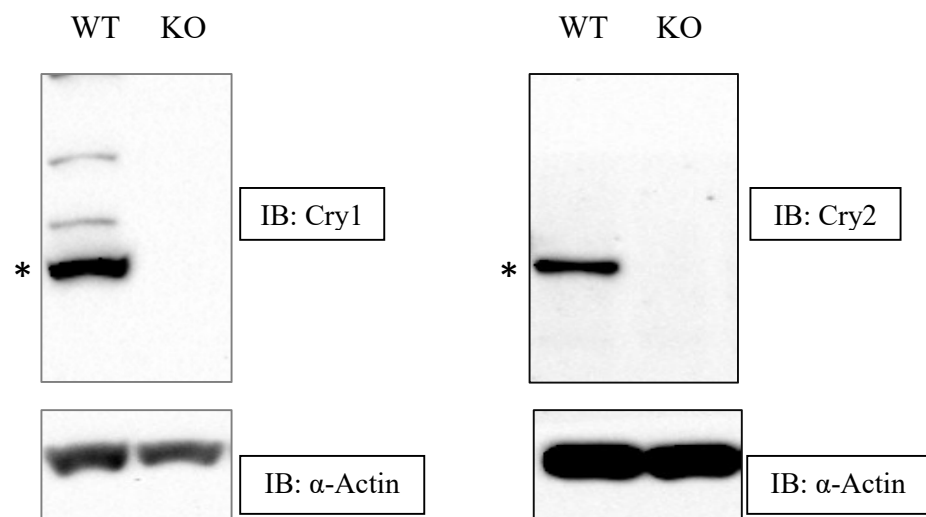


Figure 4.3: Western Blot analysis of transfected MCF10A cell extracts, Cry1: Cry2 at 10% SDS-PAGE gel. WT: Wild type cell line; KO: Knockout cell line; IB: Immunoblot; Cry1: *Cryptochrome 1* gene; Cry2: *Cryptochrome 2* gene; IB: Immunoblot.

4.2. Western Blot Analysis of Knock Outs of Clock, Bmal1 and Cry1: Cry2 Genes at Protein Level at MDA-MB-231 Cells

4.2.1. Clock Gene Screening

Western Blot screening of Clock knockout cell lines for both Wild-type Cas9 and Cas9 Nickase mutant were as shown in Figures 4.4 and 4.5. According to the results shown at Figure 4.4, *Clock* set1 and set 2 are knockouts when compared to WT cell line. In addition, at Figure 4.5., *Clock* gene is knockout when compared to WT.

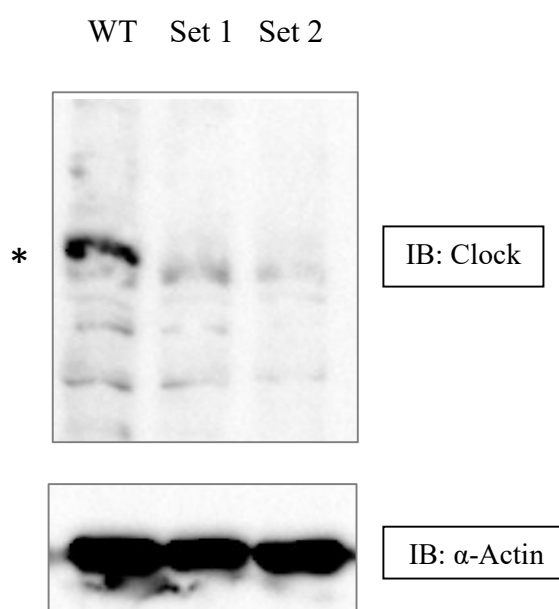


Figure 4.4: Western Blot analysis of transfected MDA-MB-231 cell extracts; Clock at 8% SDS-PAGE gel (Wild-type Cas9).
WT: Wild type cell line; Set1: Knockout 1; Set2: Knockout 2;
IB: Immunoblot.

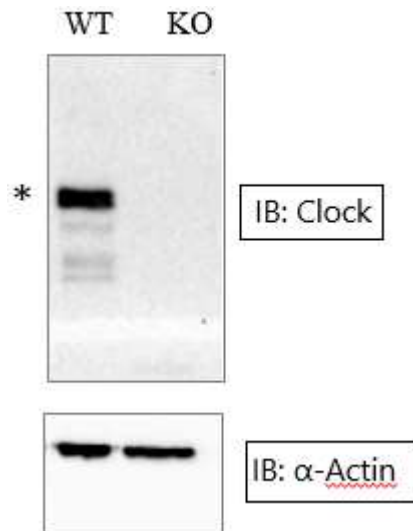


Figure 4.5: Western Blot analysis of transfected MDA-MB-231 cell extracts, Clock at 8% SDS-PAGE gel (Cas9 Nickase mutant).
WT: Wild type cell line; KO: Knockout; IB: Immunoblot.

4.2.2. Bmal1 Gene Screening

Western Blot screening of Bmal1 knockout cell lines of both Wild-type Cas9 and Cas9 Nickase mutants were as shown in Figures 4.6 and 4.7. According to the results shown at Figure 4.6, (Bmal1) set1 and set 2 are knockouts when compared to WT cell line. In addition, at Figure 4.7., Bmal1, gene is knockout when compared to WT.

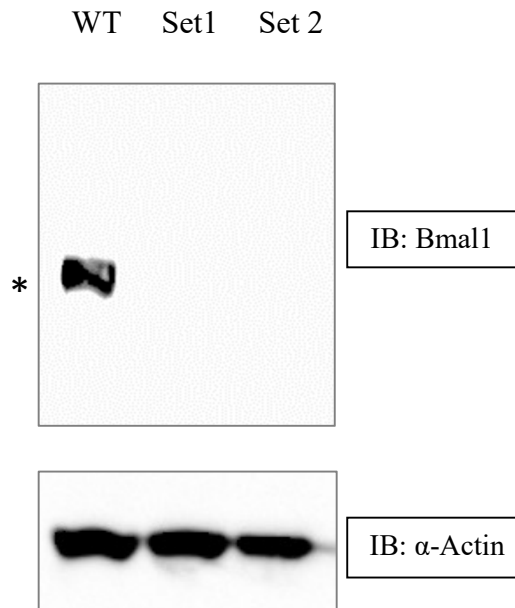


Figure 4.6: Western Blot analysis of transfected MDA-MB-231 cell extracts, Bmal1 at 8% SDS-PAGE gel (Wild-type Cas9).
WT: Wild type cell line; Set1: Knockout 1; Set2: Knockout 2;
IB: Immunoblot.

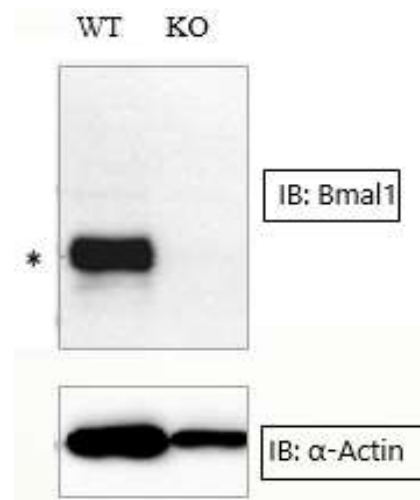


Figure 4.7: Western Blot analysis of transfected MDA-MB-231 cell extracts, Bmal1 at 8% SDS-PAGE gel (Cas9 Nickase mutant).
WT: Wild-type; KO: Knockout; IB: Immunoblot.

4.2.3. Cry1: Cry2 Gene Screening

Western Blot screening of Cry1:Cry2 knockout cell lines of both Wild-type Cas9 and Cas9 Nickase mutants were as shown in Figures 4.8 and 4.9. According to the results shown at Figure 4.8 , (Cry1) set1 and set 2 (left figure) and (Cry2) set 1 and set

2 cell lines (right figure) are knockouts when compared to WT cell (both left and right figures). In addition, at Figure 4.9., (Cry1) set1 and set 2 (left figure) and (Cry2) set 1 and set 2 cell lines (right figure) are knockouts when compared to WT cell line (both left and right figures).

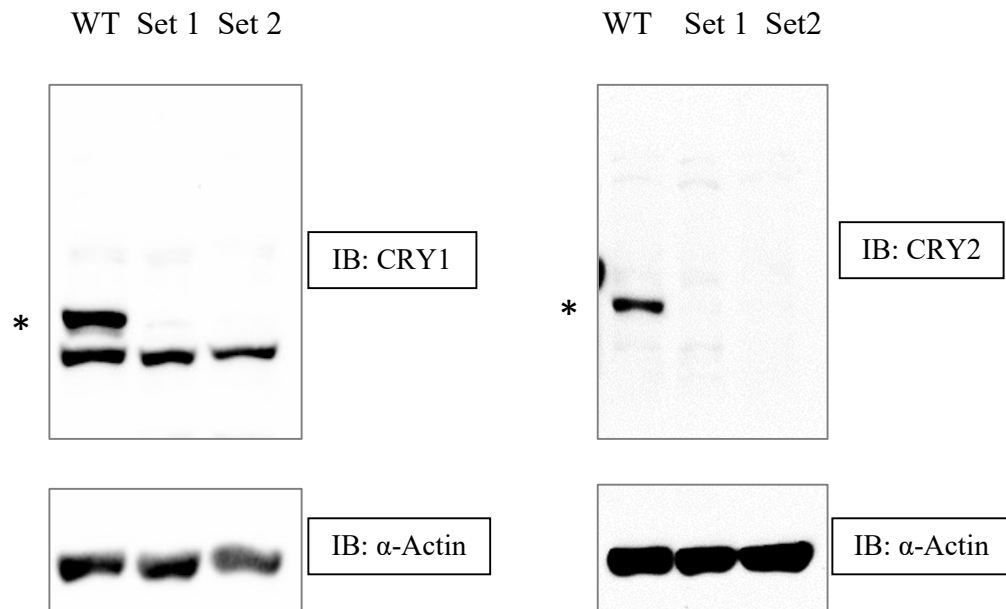


Figure 4.8: Western Blot analysis of transfected MDA-MB-231 cell extracts, Cry1: Cry2 at 10% SDS-PAGE gel (Wild-type Cas9).
 WT: Wild type cell line; Set1: Knockout 1; Set2: Knockout 2;
 IB: Immunoblot.

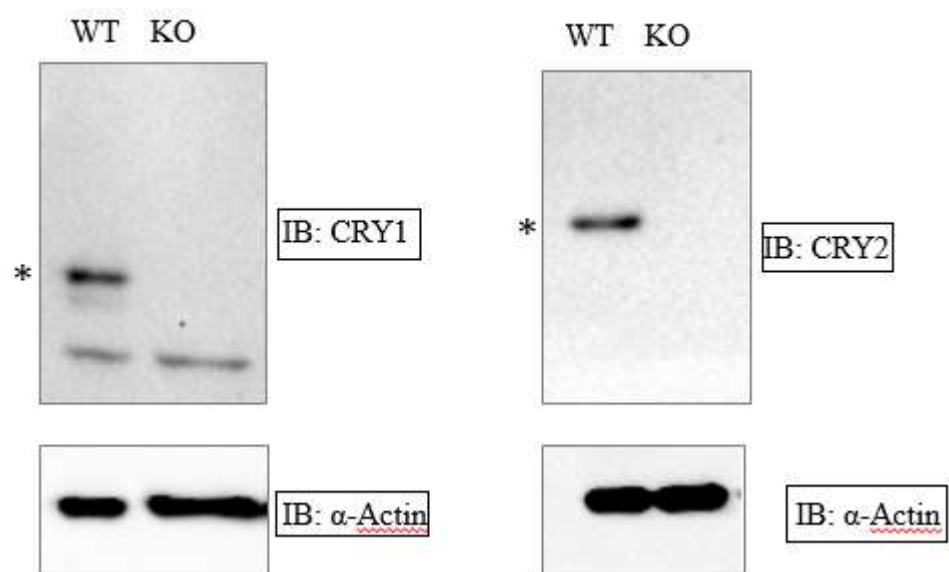


Figure 4.9: Western Blot analysis of transfected MDA-MB-231 cell extracts, Cry1: Cry2 at 10% SDS-PAGE gel (Cas9 Nickase mutant). WT: Wild Type cell line; KO: Knockout cell line; IB: Immunoblot.

4.3. Concentration and Purity of Genomic DNAs

A NucleoSpin® Tissue Kit (Macherey-Nagel) was used for DNA extraction. The results of quantification and the qualification of nucleic acids were as shown in Table 4.1.

Table 4.1: Quantification and qualification of nucleic acids

Cell-Culture Type	Knockout (KO) method	Sample Name	Subculture	Concentration (ng/μl)	260/280
MCF10A		Wild Type		87,2	1,81
	Wild Cas 9-mediated	Clock KO	-	103	1,84
		Bmal1 KO	Set1	26,7	1,71
			Set2	129,3	1,85
		CRY1/2 KO	-	108,5	1,86
MDAM231		Wild Type		44,67	1,84
	Nickase Cas 9-mediated	Clock KO	-	49,71	1,87
		Bmal1 KO	-	30,49	1,89
		CRY1/2 KO	-	53,86	1,87
		Wild Type		665	2,01
	Wild Cas 9-mediated	Clock KO	Set1	314,8	1,98
			Set2	594,2	1,93
		Bmal1 KO	Set1	326,9	1,91
			Set2	405	2,02
		CRY1/2 KO	Set1	596,3	1,99
			Set2	500	1,97

Following DNA extraction, DNAs were run on a 0.8% agarose gel, as shown in Figure 4.10. All genomic DNAs were obtained as an intact.

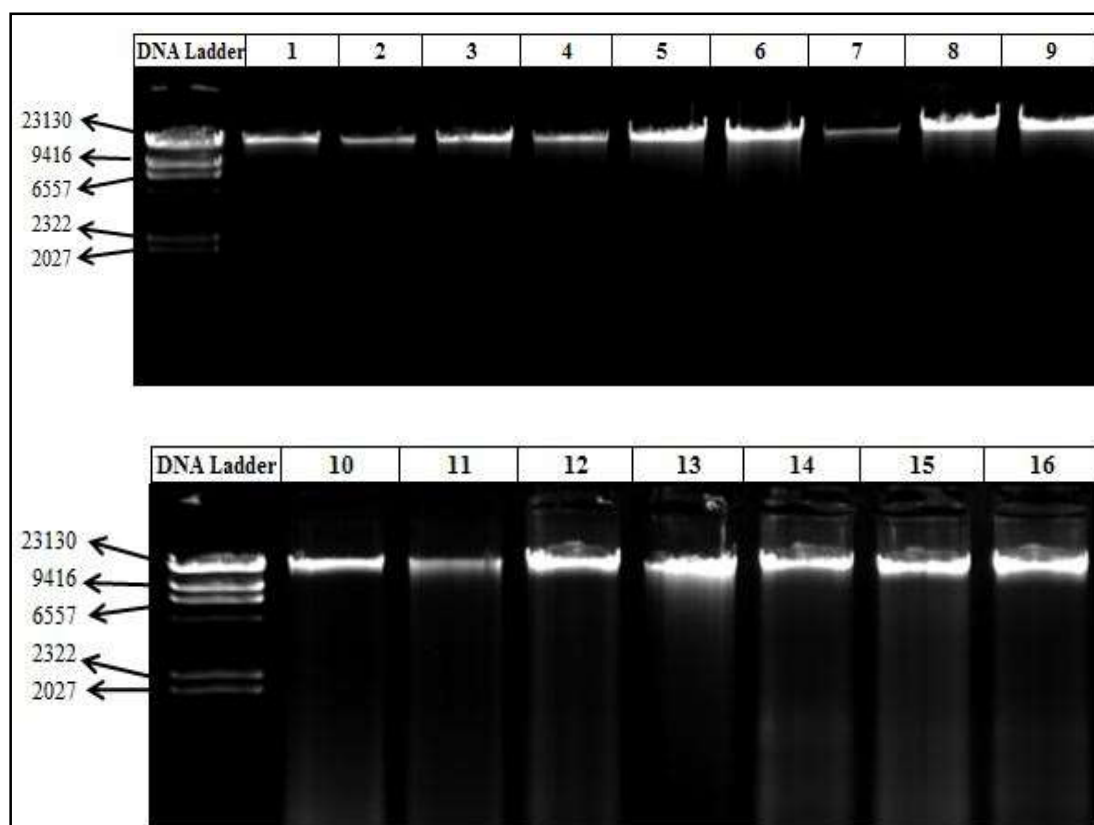


Figure 4.10: Agarose gel electrophoresis of extracted DNA samples. DNAs were loaded and analysed by agarose gel (0.8%) electrophoresis and visualised under UV light. Lanes: DNA ladder- GeneRuler λ Hind III Marker; lane 1: MCF10A-Wild-type; lanes 2: MCF10A *Clock* KO; lanes 3 and 4: MCF10A- *Bmal1* KO Set1 and Set 2; lane 5: MCF10A- *CRY 1&2* KO; lane 6: MDA-MB-231- Wild-type; lane 7: MDA-MB-231- *Clock* KO (Nickase Cas 9-mediated); lane 8: MDA-MB-231- *Bmal1* KO (Nickase Cas 9-mediated); lane 9: MDA-MB-231-*CRY 1&2* KO (Nickase Cas 9-mediated); lane 10: MDA-MB-231-Wild-type; lanes 11 and 12: MDA-MB-231- *Clock* KO Set 1 and Set 2 (Wild-type Cas 9-mediated); lanes 13 and 14: MDA-MB-231- *Bmal1* KO Set 1 and Set 2 (Wild-type Cas 9-mediated); lanes 15 and 16: MDA-MB-231- *CRY 1&2* KO Set 1 and Set 2 (Wild-type Cas 9-mediated).

4.4. High Resolution Melting (HRM) Dye Optimization

High Resolution Melting (HRM) dye optimization was performed after bisulfite treatment. Human Methylated and Non-methylated DNA Set was studied for HRM dye optimization. The results of different HRM dye kits were as shown in Figure 4.11. ZymoTaq™ qPCR Premix gave the best melting peaks, other master mixes showed a shoulder structure. Whether decided the structure is shoulder structure or non-specific binding, DNAs were run on a 2% agarose gel. This structure is a shoulder structure, not a non-specific binding shown in Figure 4.12.

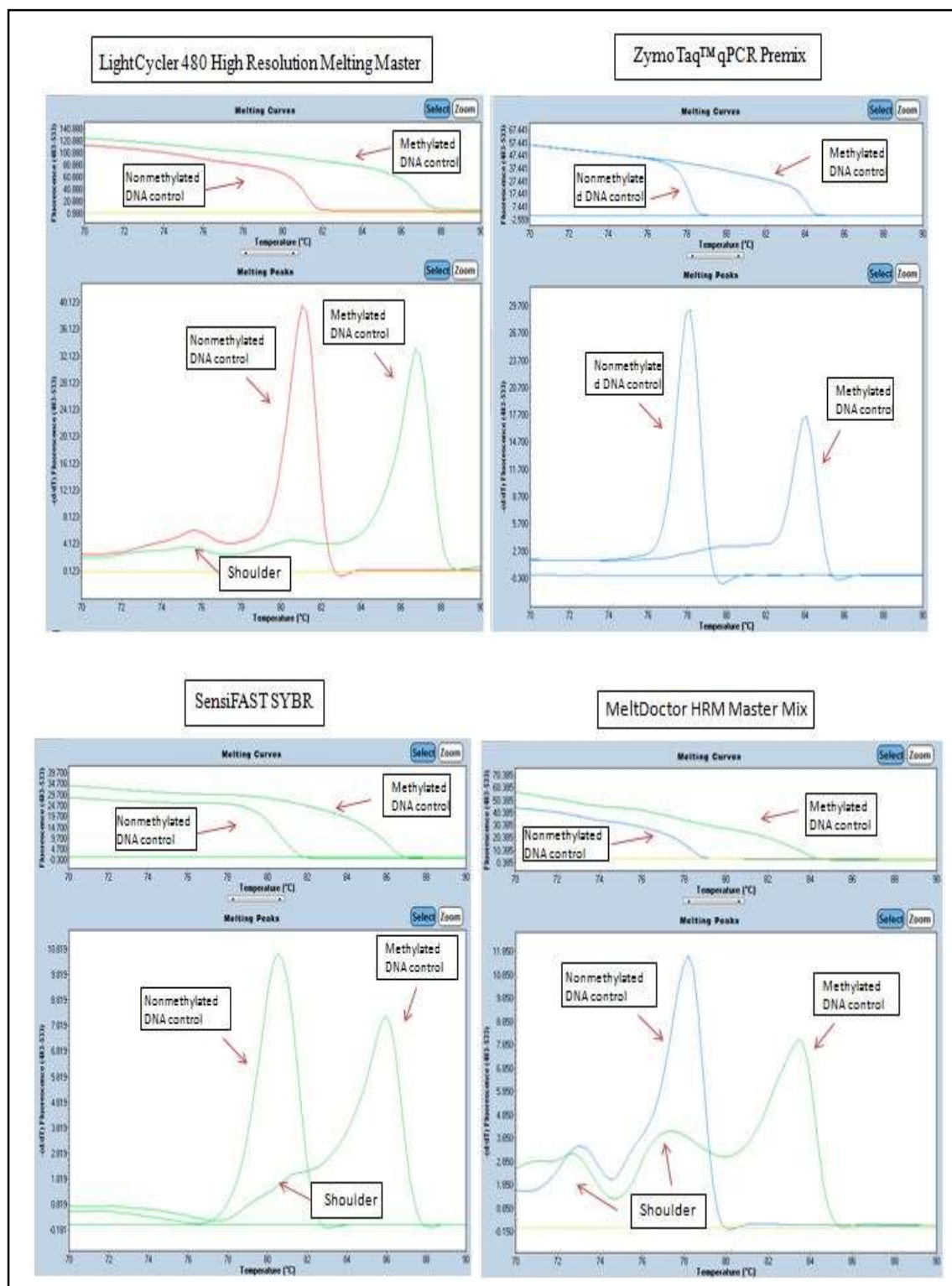


Figure 4.11: Melting curve and melting peak views of different HRM dyes. LightCycler 480 High Resolution Melting Master, MeltDoctor HRM Master Mix and SensiFAST SYBR master mixes showed shoulders.

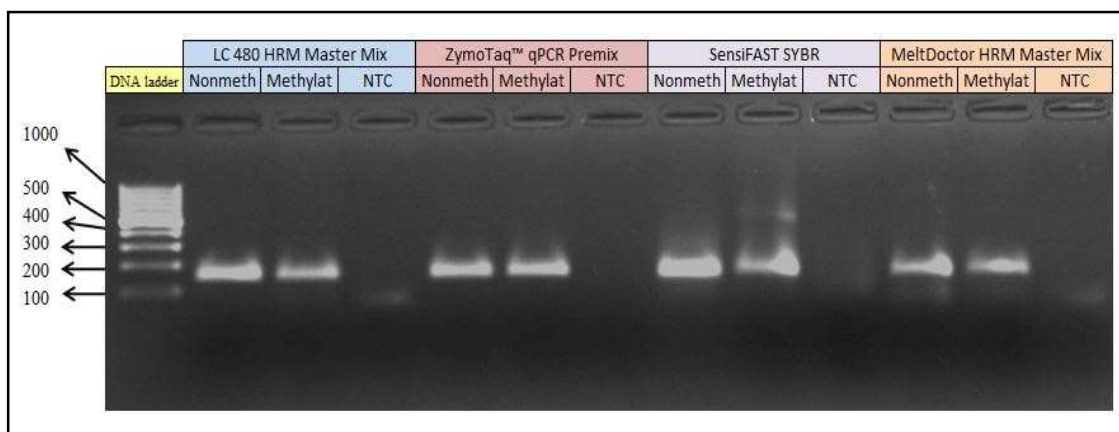


Figure 4.12: Agarose gel view of PCR with different commercial dyes. NTC: non-template control. (The DNA ladder is 100–1,000 kb)

4.5. High Resolution Melting (HRM) Analysis

BRCA1, RASSF1A and RAR β genes PCR products were exposed to HRM, in the 40–95°C melting temperature range. The temperature that resulted in the clearest and sharpest peak was chosen as the optimum annealing temperature and the optimum melt temperature range was determined. Each sample was studied twice but shown once in a graph.

The Human Methylated and Non-methylated DNA Set was studied with DAPK1 primers to check the bisulfite conversion. Melting curves and melting peaks were as shown in Figure 4.13.

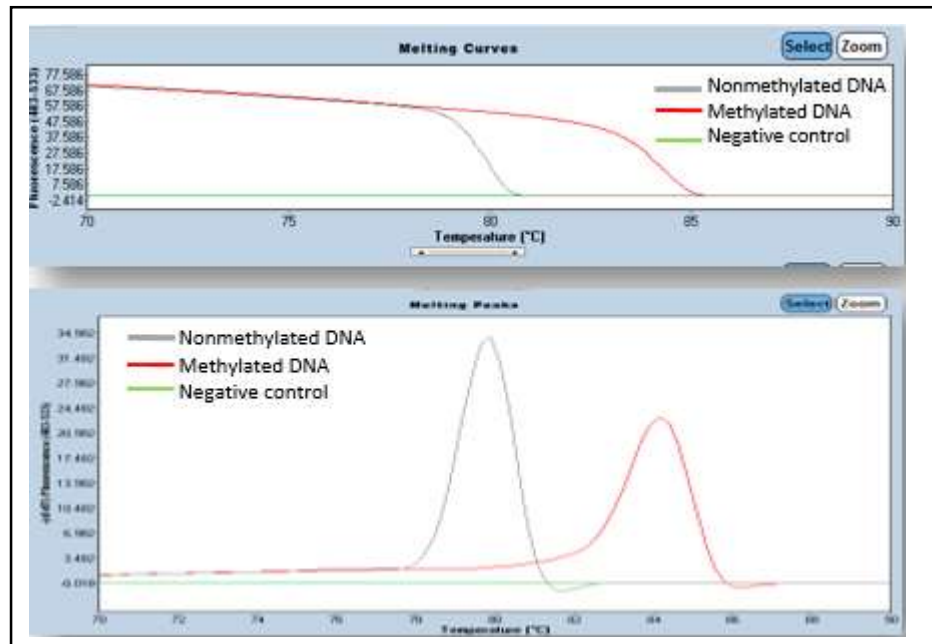


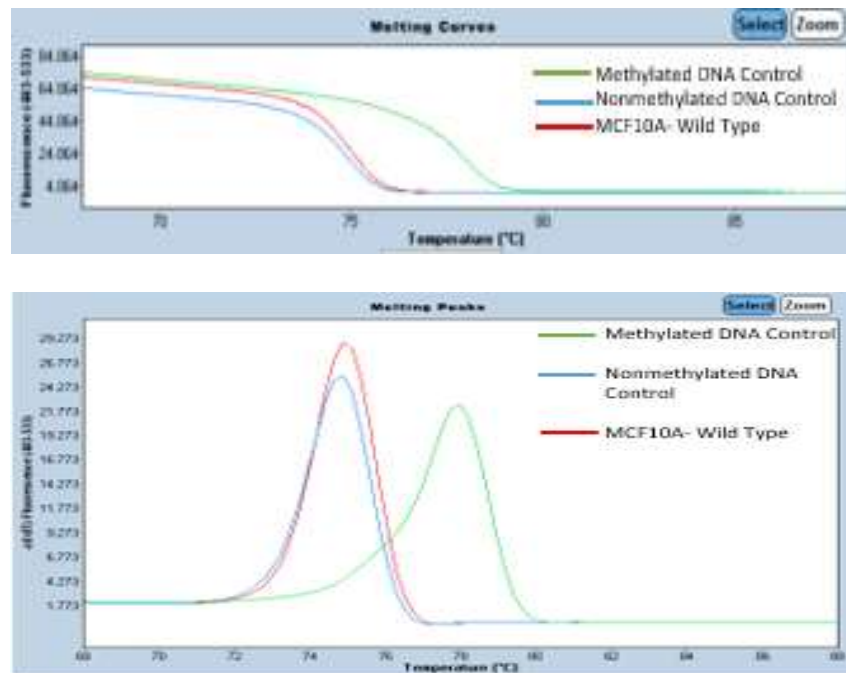
Figure 4.13: Checking bisulfite treatment. The Human Methylated and Non-methylated DNA Set (Zymo Research) was used as a control sample. Together with MCF10A and MDA-MB-231 cell lines, the Human Methylated and Non-methylated DNA Set was also treated with bisulfite conversion. Conversion accuracy was checked with DAPK1 primers.

4.5.1. High-resolution Melting (HRM) Analysis of the *BRCA1* gene

As a result of HRM analysis with BRCA primers, all samples were found to give specific HRM curves. MCF10A cell line and knockouts of MCF10A cell lines were studied with BRCA1 primers and the obtained HRM profiles are given in Figure 4.14 (a–f).

Similarly, MDAMB231 cell lines and their CRISPR knockouts (both Nickase Cas 9- and Wild-type Cas 9-mediated) were studied with BRCA1 primers and the obtained HRM profiles are given in Figure 4.15 (a–d) and Figure 4.16 (a–g). HRM analysis of all samples were given in Figure 4.17.

a)



b)

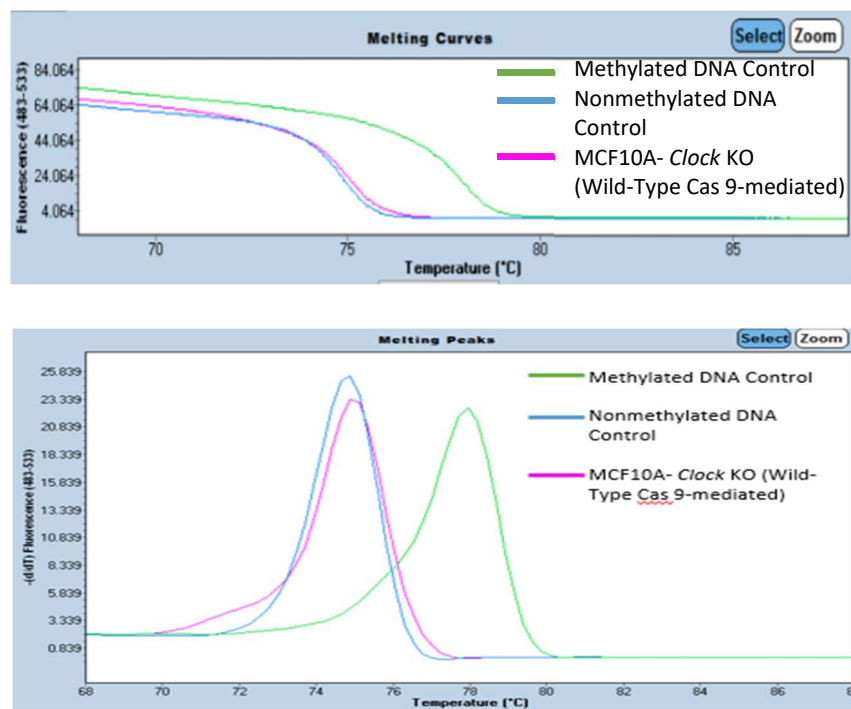
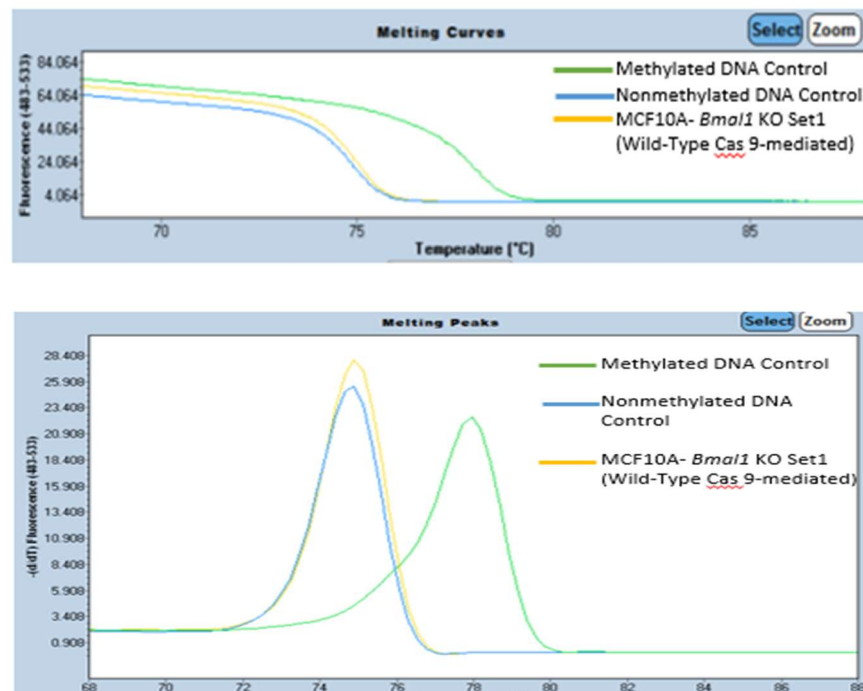


Figure 4.14: BRCA1 melting curves and melting peaks of the MCF10A cell line and its knockouts. a) MCF10A wild-type cell line HRM profile. b) MCF10A *Clock* knockout cell line HRM profile. c -d) MCF10A *Bmal1* knockout cell line Set 1 and Set 2 HRM profile, respectively. e) MCF10A *CRY 1&2* knockout cell line HRM profile. f) Negative control.

c)



d)

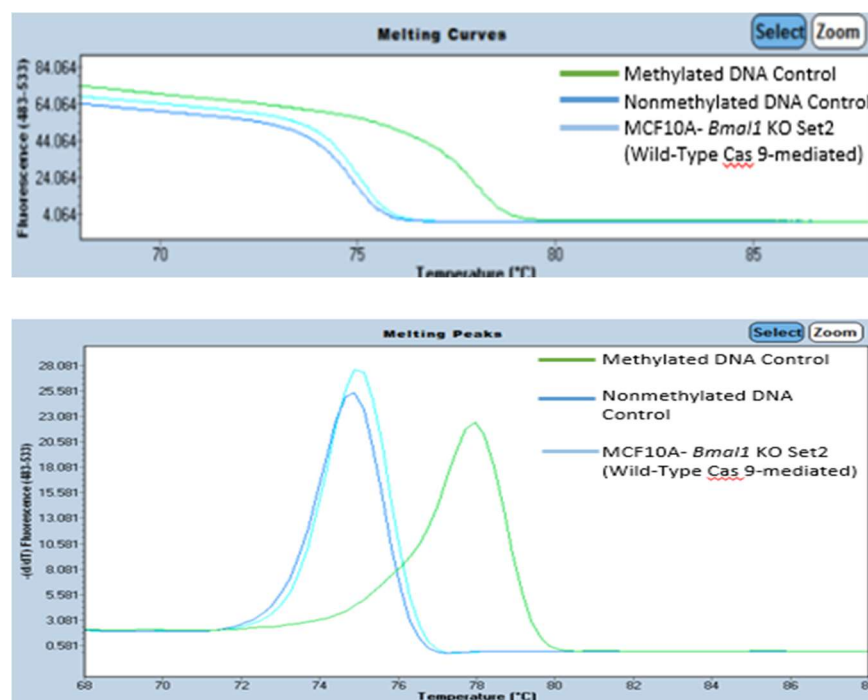
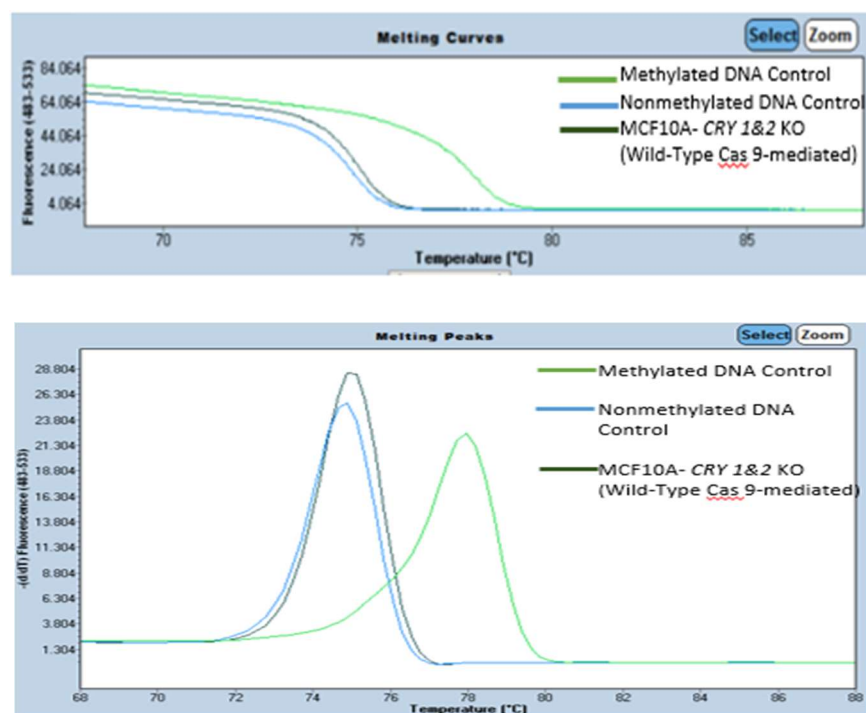


Figure 4.14: Continued

e)



f)

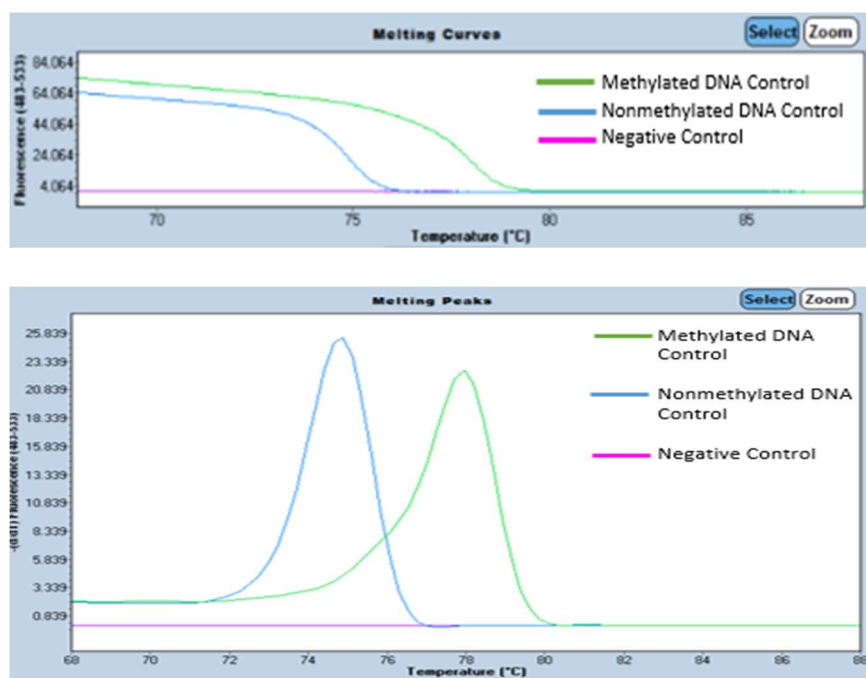
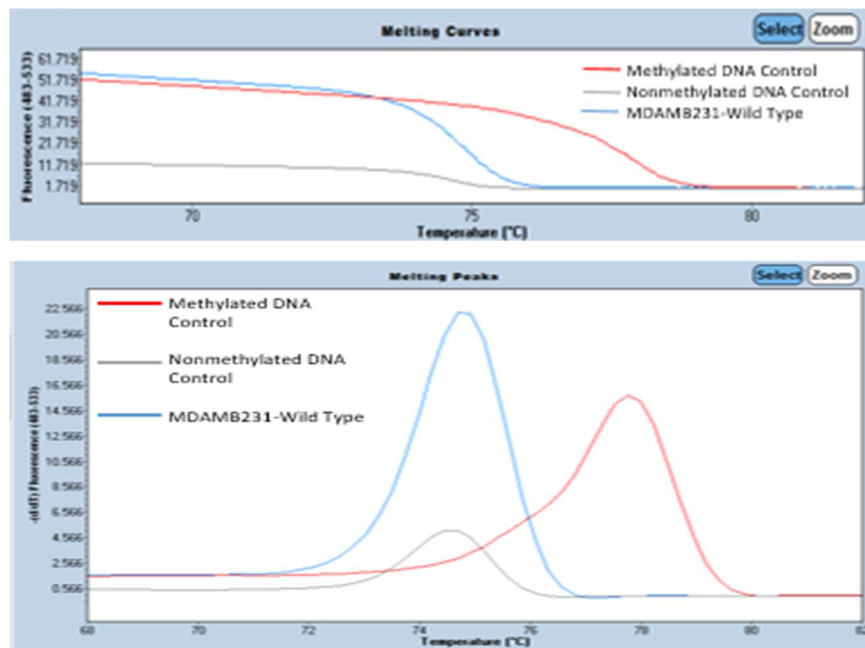


Figure 4.14: Continued

a)



b)

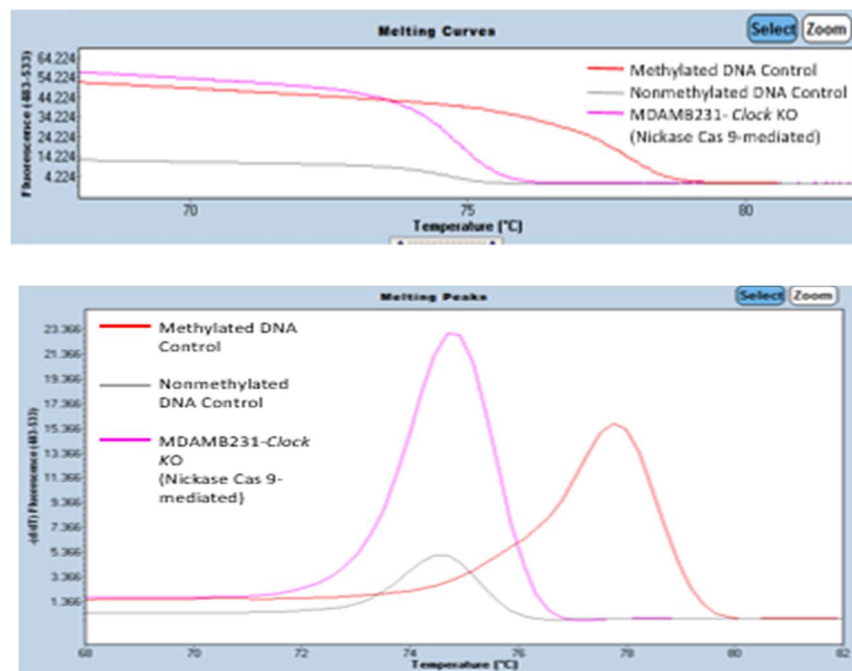
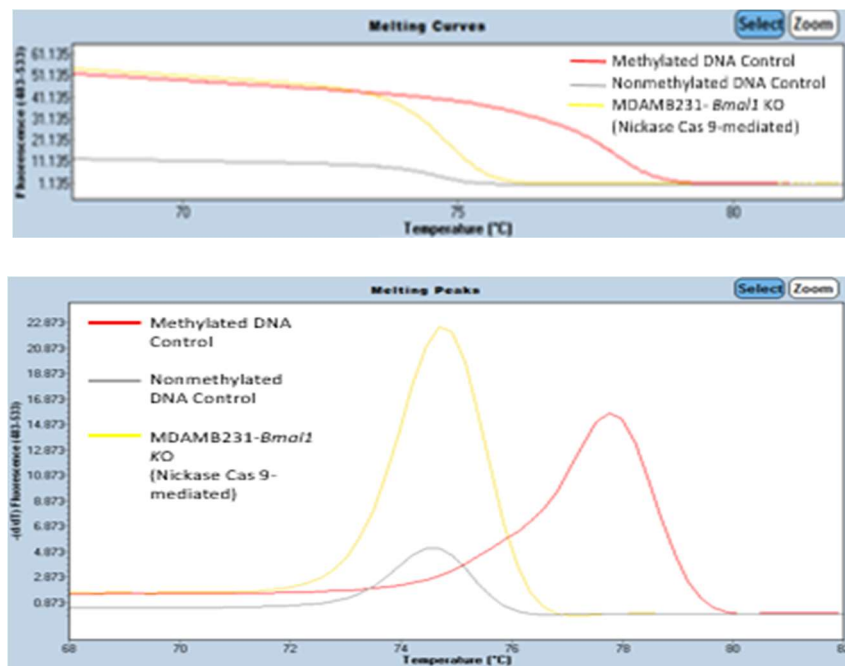


Figure 4.15: BRCA1 melting curves and melting peaks of MDA-MB-231 cell line and its knockouts with Nickase Cas9 knockouts. (a) MDA-MB-231 wild-type cell line HRM profile. (b) MDA-MB-231 *Clock* knockout cell line (Nickase Cas9) HRM profile. (c) MDA-MB-231 *Bmal1* knockout cell line (Nickase Cas9) HRM profile. (d) MDA-MB-231 *CRY 1&2* knockout cell line (Nickase Cas9) HRM profile.

c)



d)

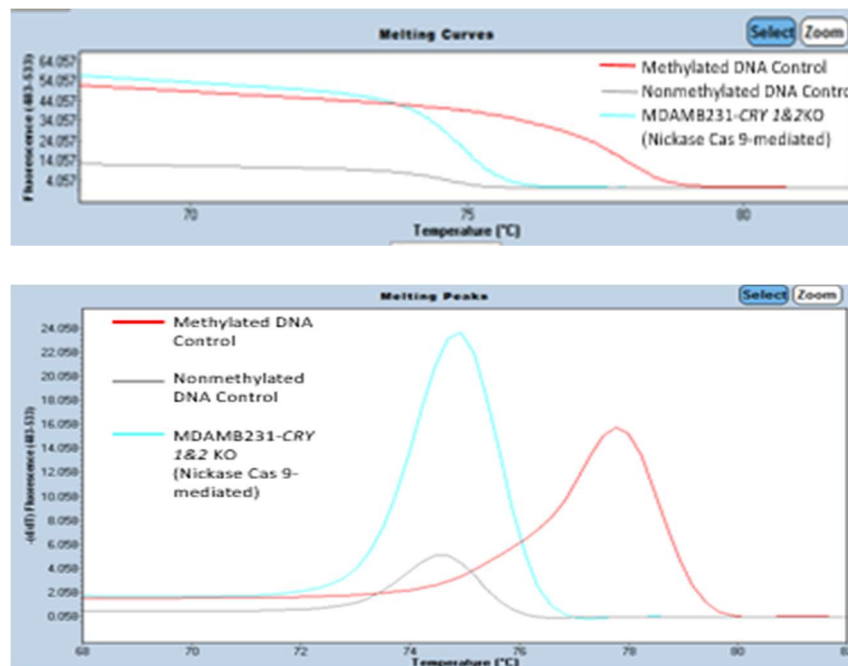
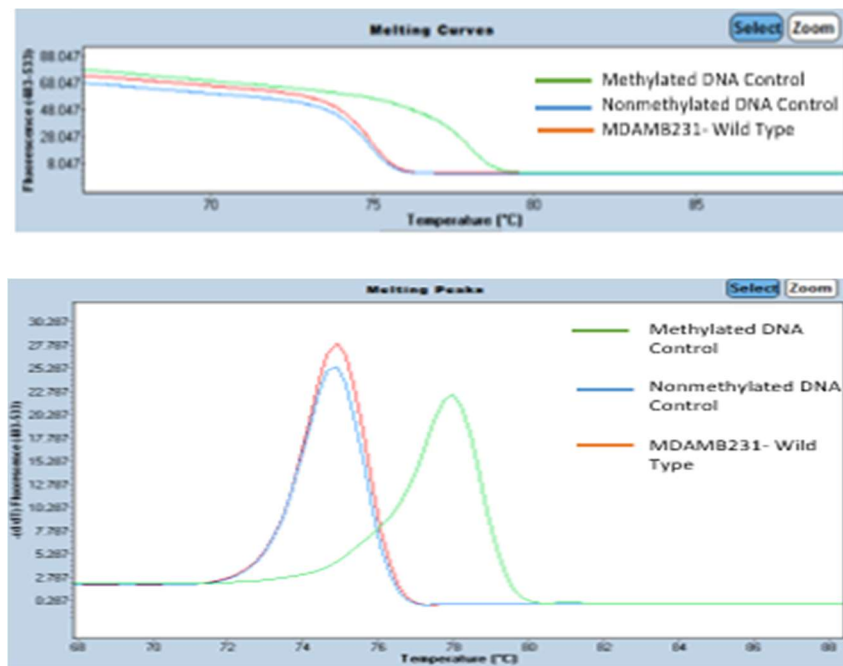


Figure 4.15: Continued

a)



b)

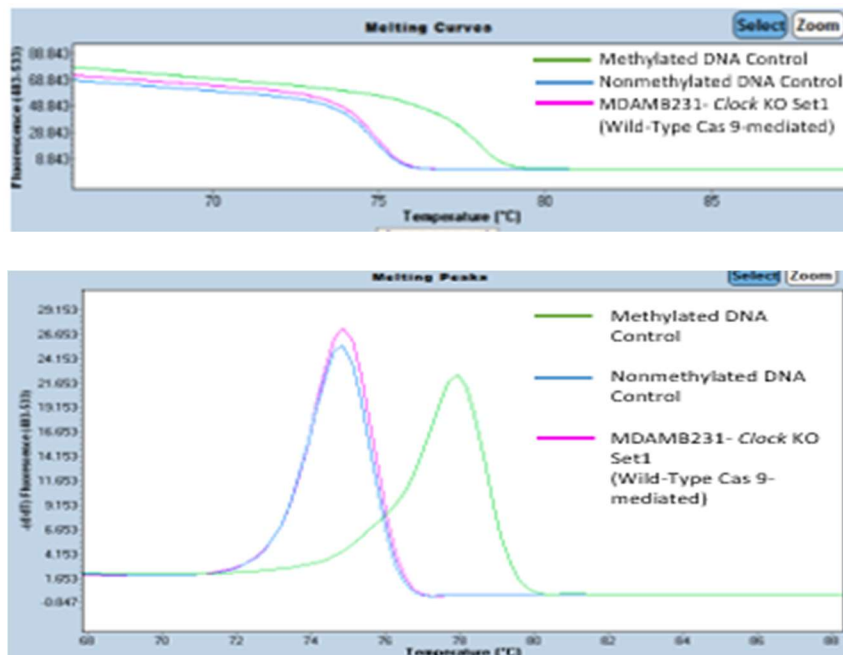
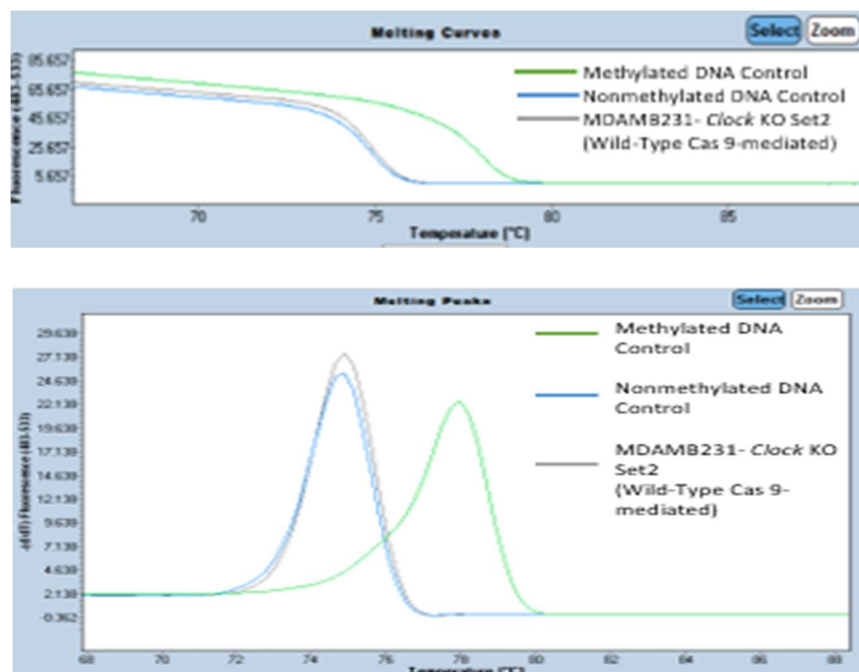


Figure 4.16: BRCA1 melting curves and melting peaks of the MDA-MB-231 cell line and its knockouts with Wild-type Cas9. a) MDA-MB-231 wild-type cell line HRM profile. b-c) MDA-MB-231 *Clock* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profiles, respectively. d-e) MDA-MB-231 *Bmal1* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively. f-g) MDA-MB-231 *CRY1* & 2 knockout cell lines (Wild-type Cas9) Set1 and Set2 HRM profile, respectively.

c)



d)

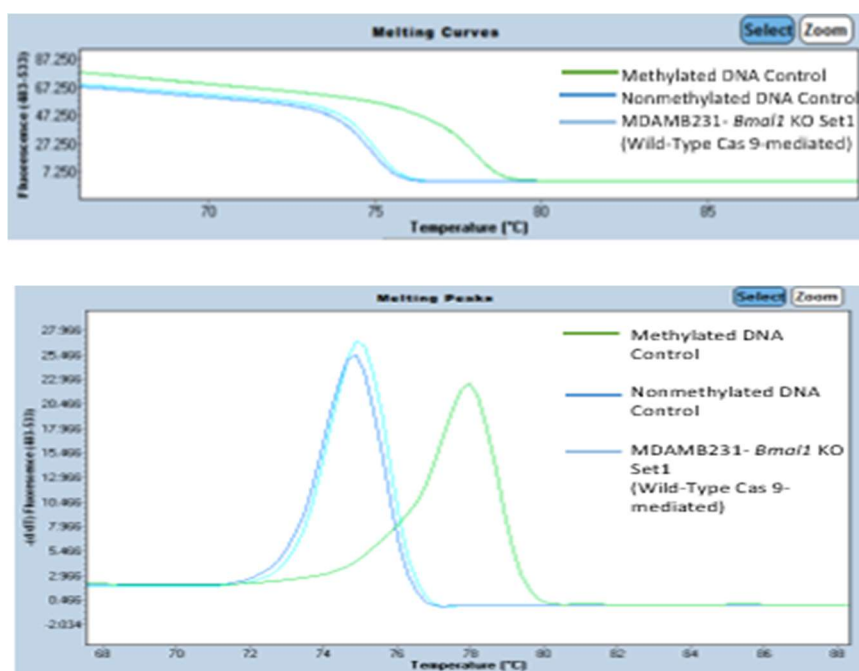
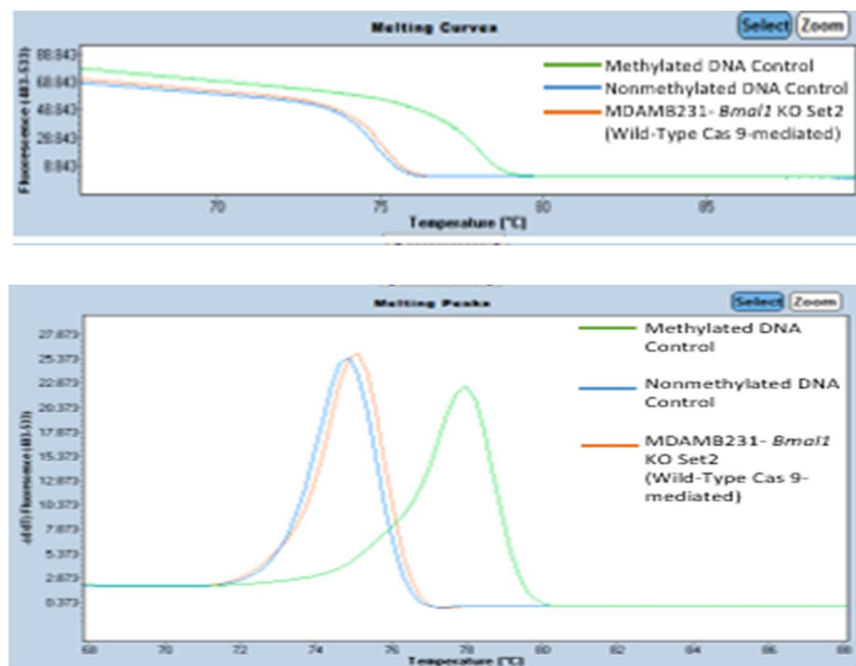


Figure 4.16: Continued

e)



f)

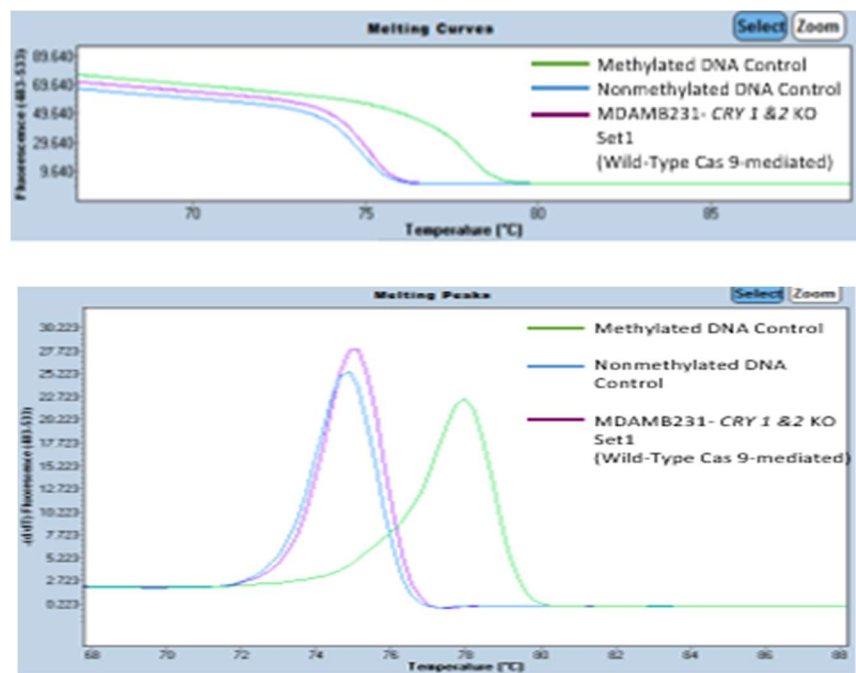


Figure 4.16: Continued

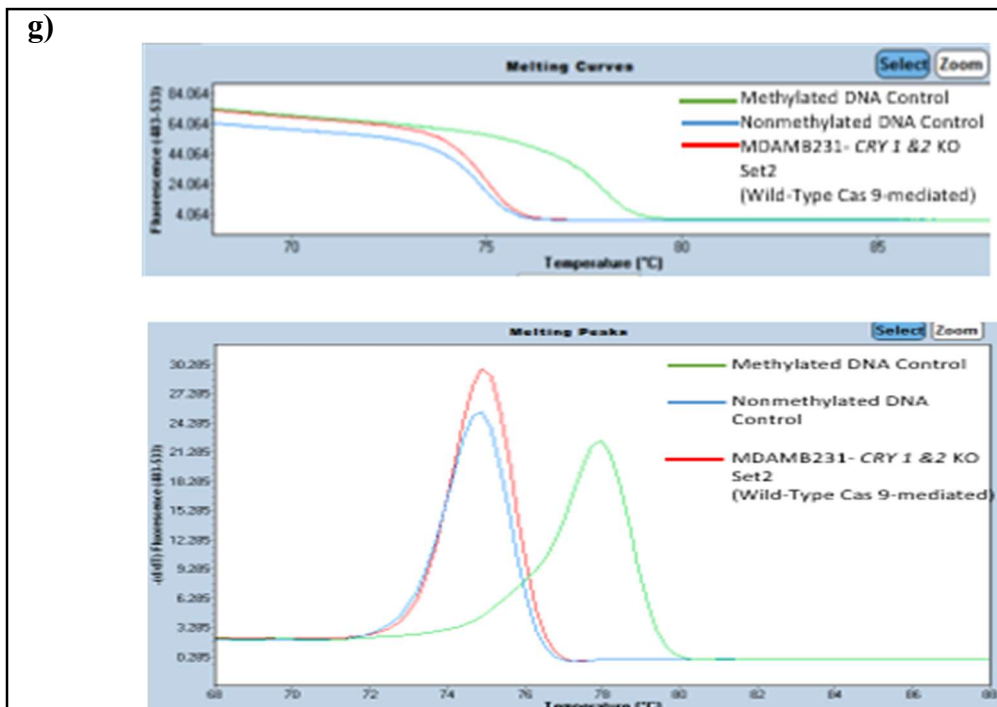


Figure 4.16: Continued

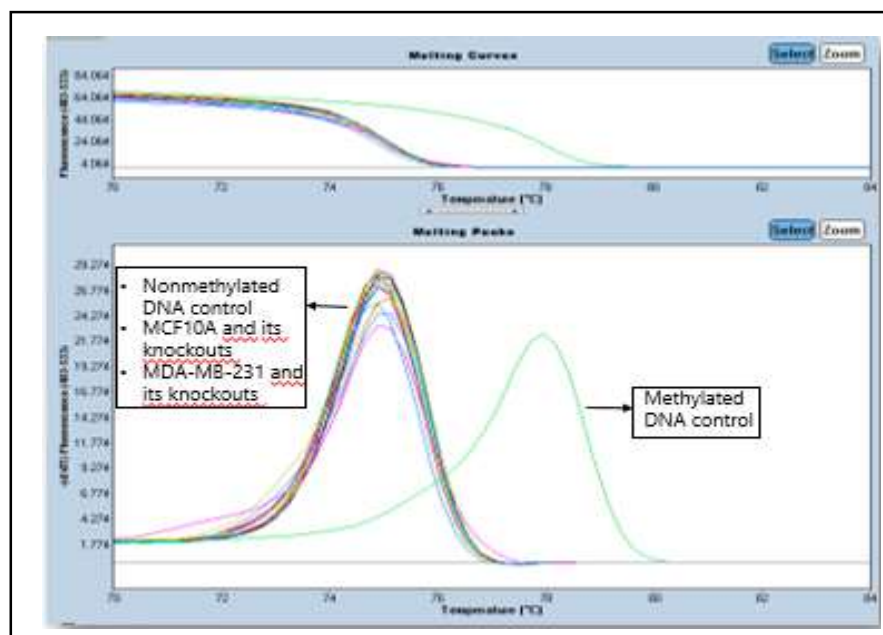


Figure 4.17: BRCA1 melting curves and melting peaks of both MCF10A and MDA-MB-231 cell lines and their knockouts.

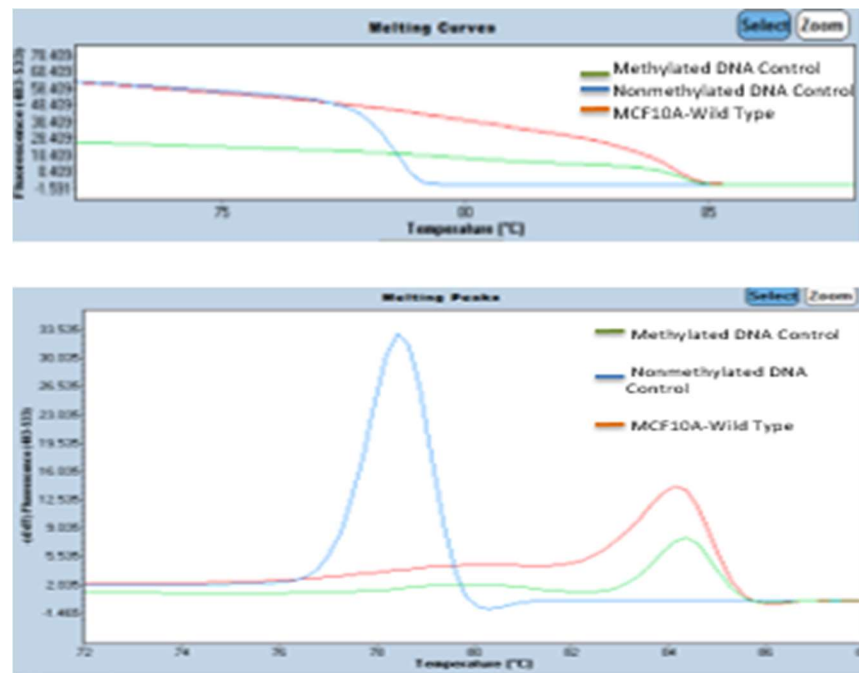
Nonmethylated DNA control have more low melting temperature than methylated control because after bisulfite treatment nonmethylated cytosine converts to uracil and recognize by thymine following PCR amplification. Both of them, uracil and thymine, make a two-hydrogen bond. On the other hands, methylated cytosine does not convert to uracil and remain as a cytosine. This cytosine makes a three-hydrogen bond with guanine following PCR amplification. Therefore, methylated DNA control have more high melting temperature than nonmethylated DNA control. Figure 4.14a shows the MCF10A cell line has shown a non-methylated profile because it displayed same melting curve and melting peaks with nonmethylated DNA control. The MCF10A cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.14.b–e). Figures 4.15.a and 4.17 shows the MDA-MB-231 cell line has shown a non-methylated profile because it displayed same melting curve and melting peaks with nonmethylated DNA control. The MDA-MB-231 cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.15.b–e and 4.16b–g). Figure 4.17 showed that all samples including methylated and non-methylated DNA controlled the *BRCA1* gene methylation profile.

4.5.2. High-resolution Melting (HRM) Analysis of *RASSF1A* gene

The results of HRM analysis with *RASSF1A* primers showed that all samples gave specific HRM curves. The MCF10A cell line and knockouts of the MCF10A cell lines were studied with *RASSF1A* primers and obtained HRM profiles were given in Figure 4.18.

Similarly, MDAMB231 cell lines and their CRISPR knockouts (both Nickase Cas9-mediated and Wild-type Cas9-mediated) were studied with *RASSF1A* primers and the obtained HRM profiles are given in Figures 4.19 and 4.20. The HRM analyses of all samples are given in Figure 4.21.

a)



b)

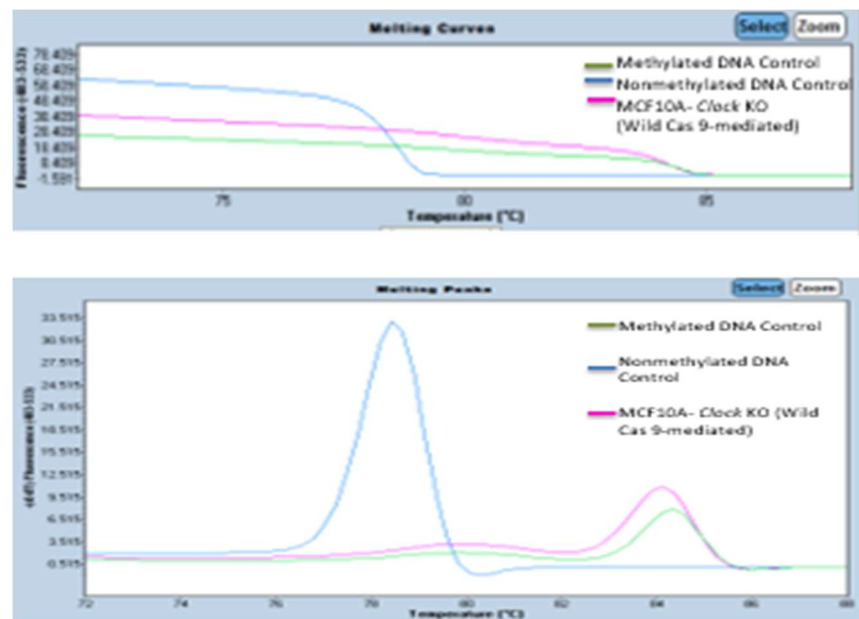
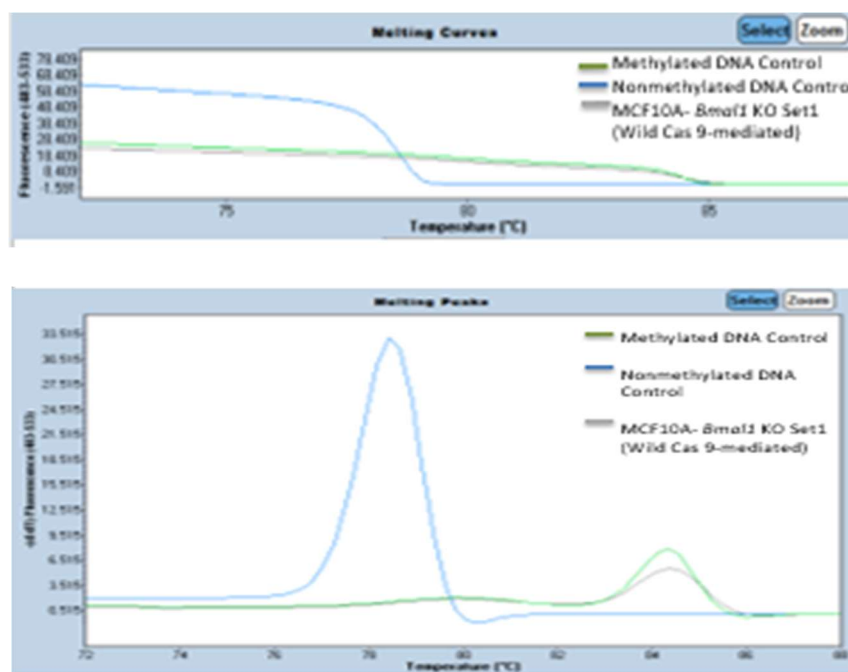


Figure 4.18: RASSF1A melting curves and melting peaks of the MCF10A cell line and its knockouts with Wild-type Cas9. a) MCF10A wild-type cell line HRM profile. b) MCF10A *Clock* knockout cell line HRM profile. c-d) MCF10A *Bmal1* knockout cell line Set1 and Set2 HRM profile, respectively. e) MCF10A *CRY 1&2* knockout cell line HRM profile. f) Negative control.

c)



d)

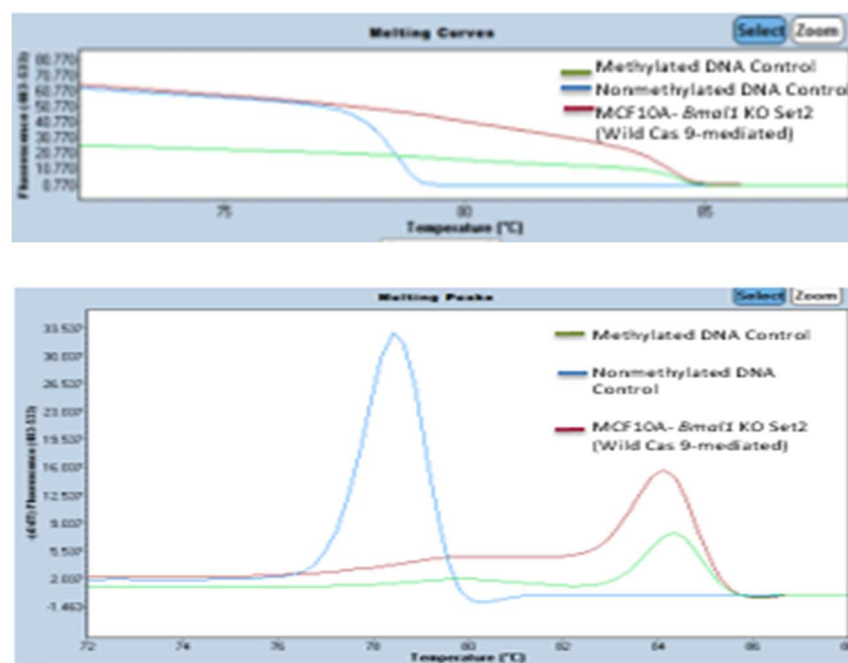
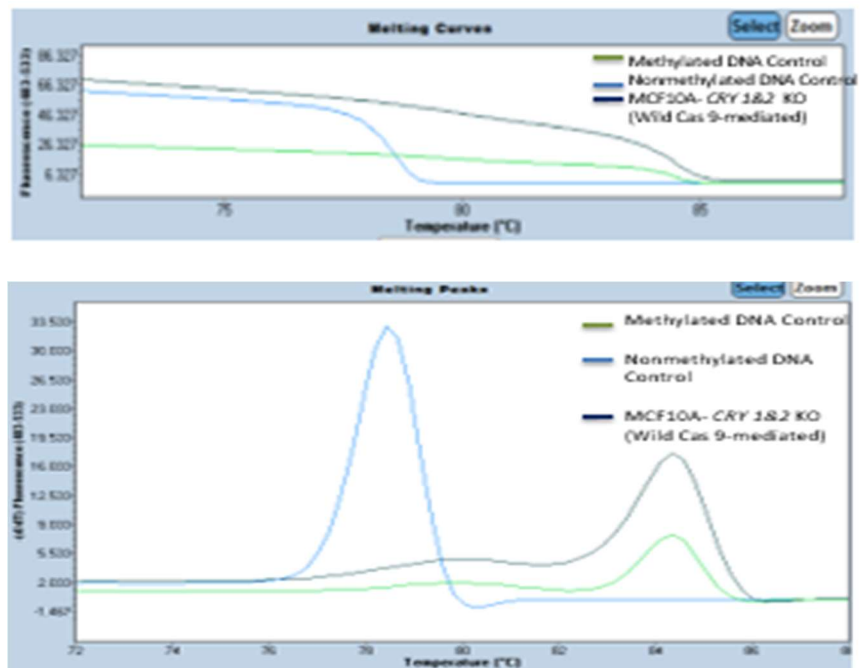


Figure 4.18: Continued

e)



f)

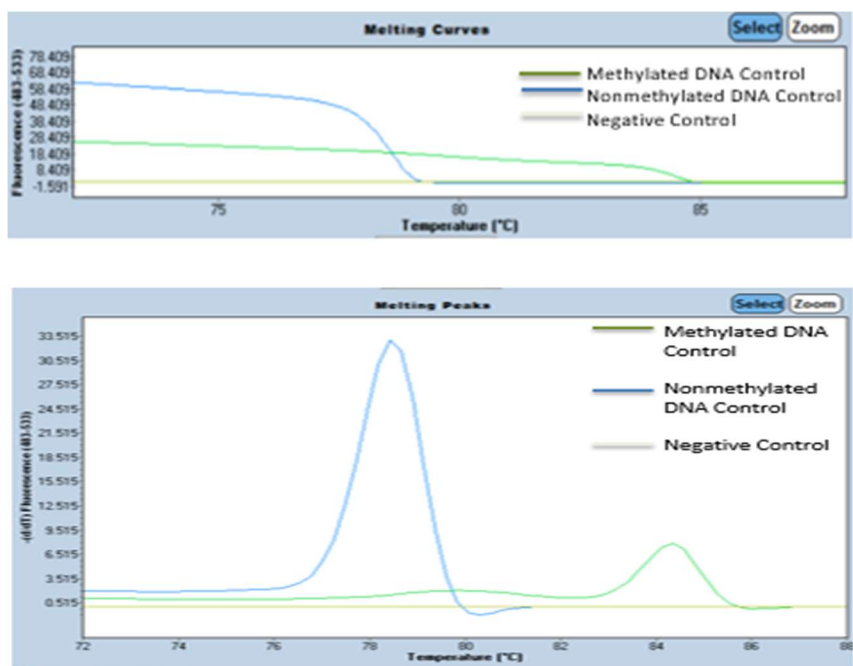


Figure 4.18: Continued

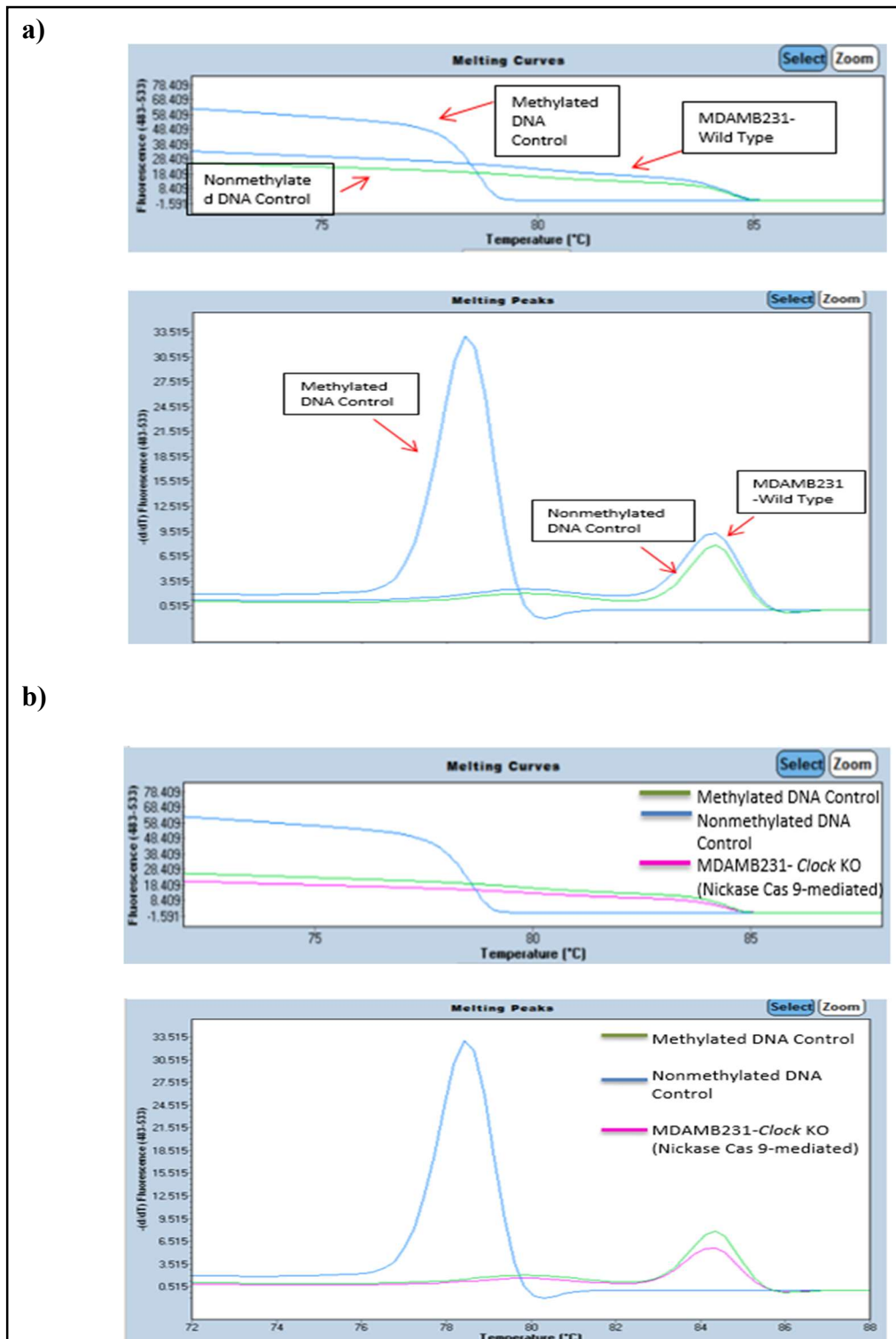


Figure 4.19: RASSF1A melting curves and melting peaks of MDA-MB-231 cell line and its knockouts with Nickase Cas9. a) MDA-MB-231 wild-type cell line HRM profile. b) MDA-MB-231 *Clock* knockout cell line (Nickase Cas9) HRM profile. c) MDA-MB-231 *Bmal1* knockout cell line (Nickase Cas9) HRM profile d) MDA-MB-231 *CRY 1&2* knockout cell line (Nickase Cas9) HRM profile.

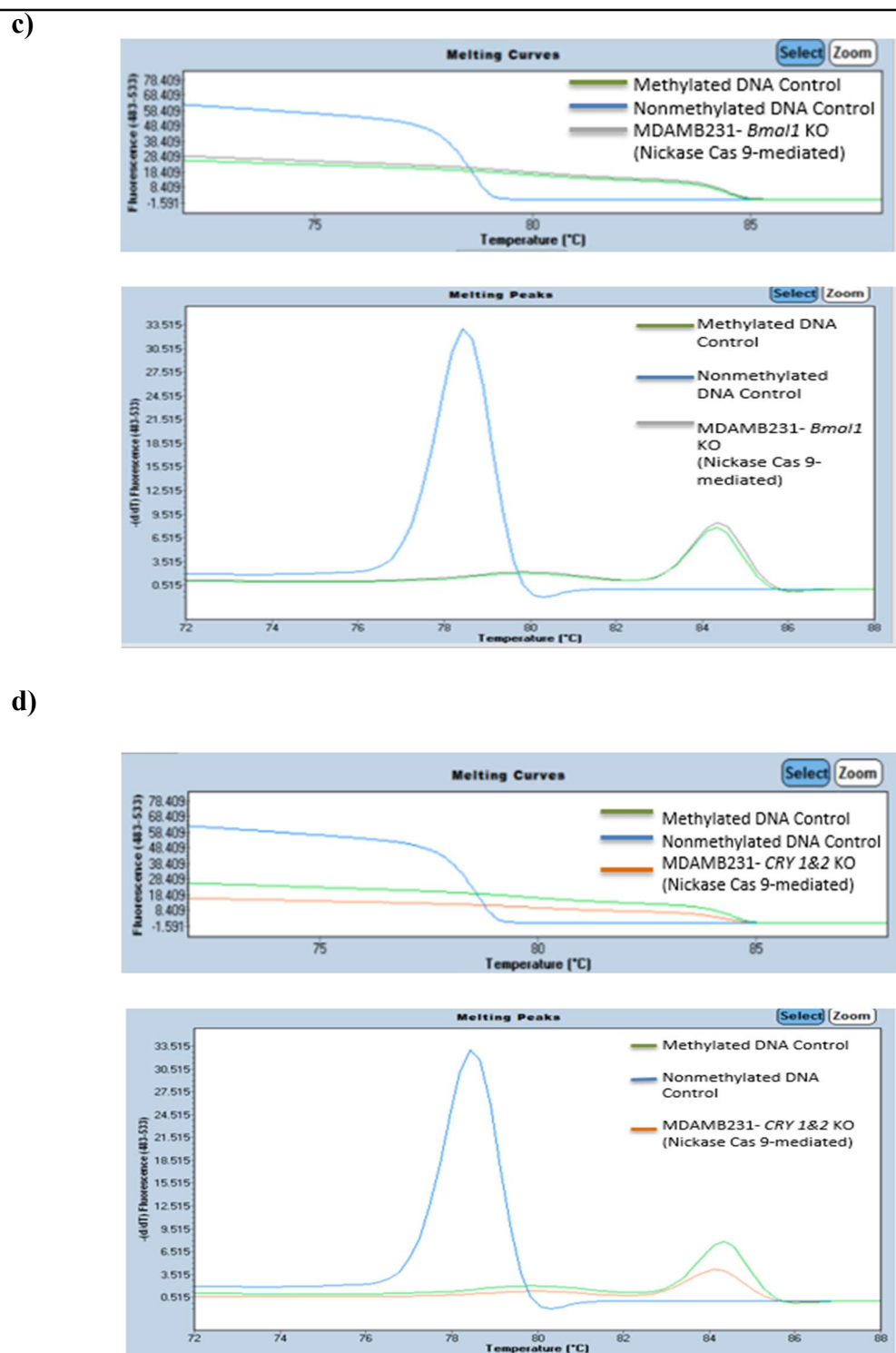


Figure 4.19: Continued

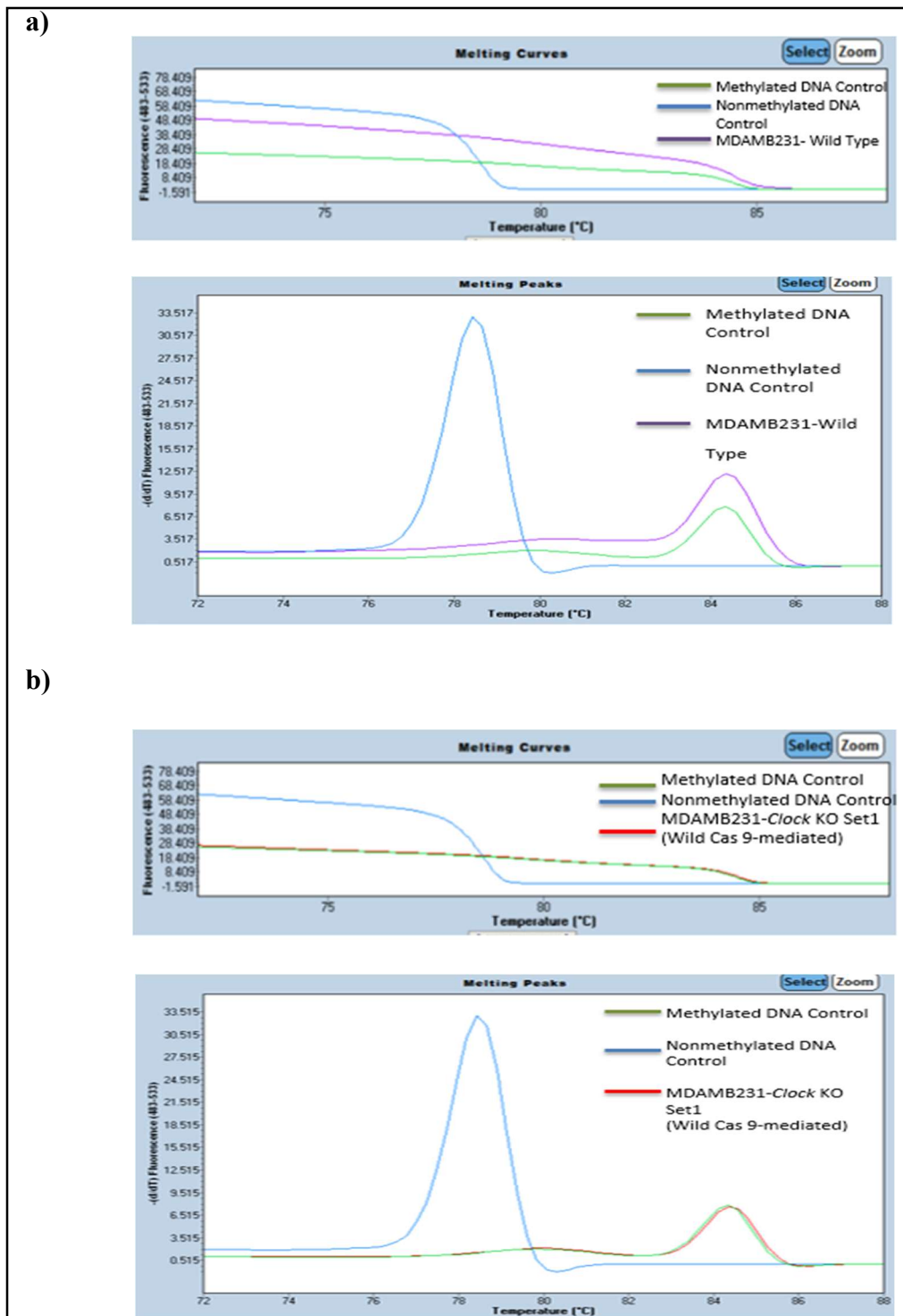
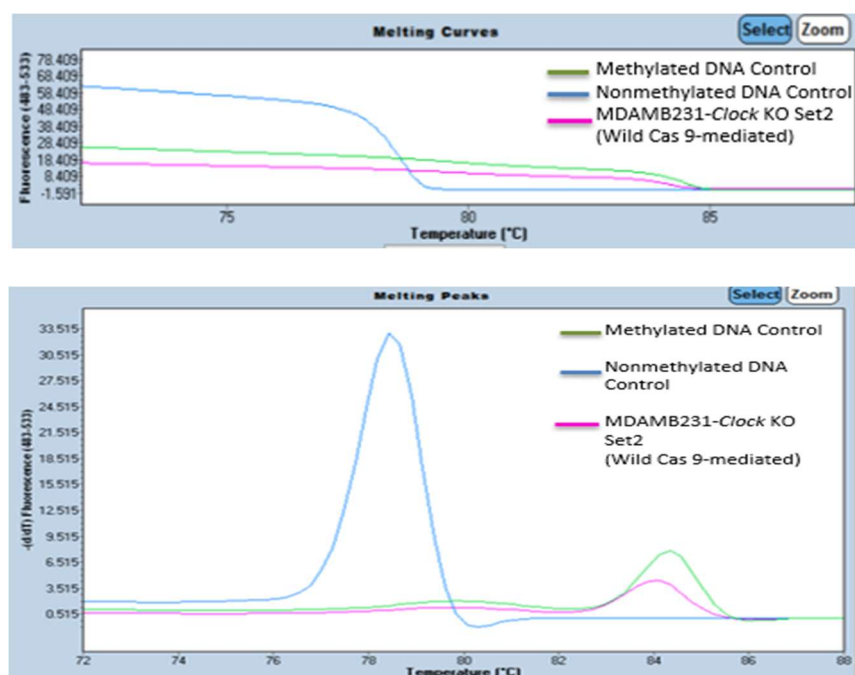


Figure 4.20: RASSF1A melting curves and melting peaks of MDA-MB-231 cell line and its knockouts with Wild-type Cas9. a) MDA-MB-231 wild-type cell line HRM profile. b-c) MDA-MB-231 *Clock* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively. d-e) MDA-MB-231 *Bmal1* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively. f-g) MDA-MB-231 *CRY 1&2* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively.

c)



d)

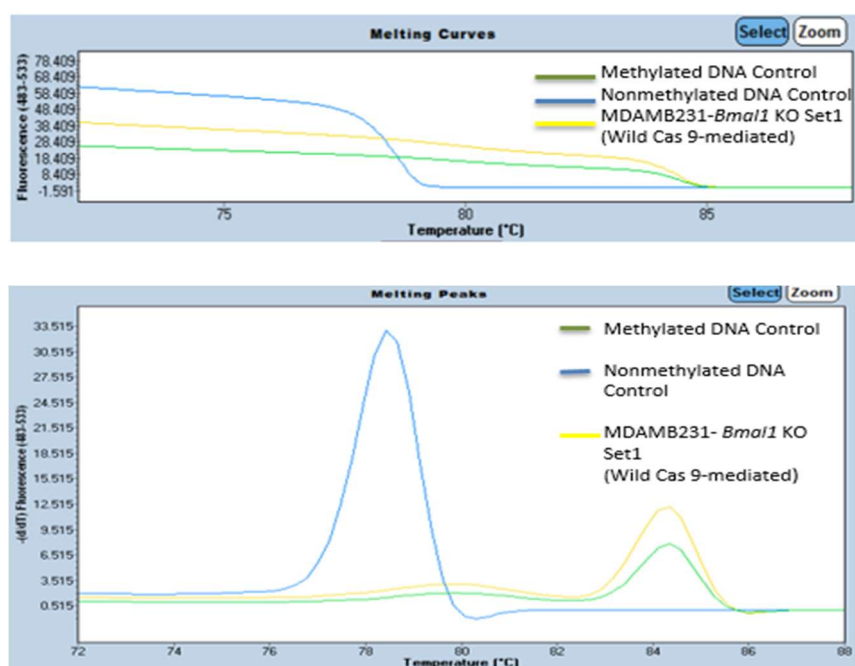
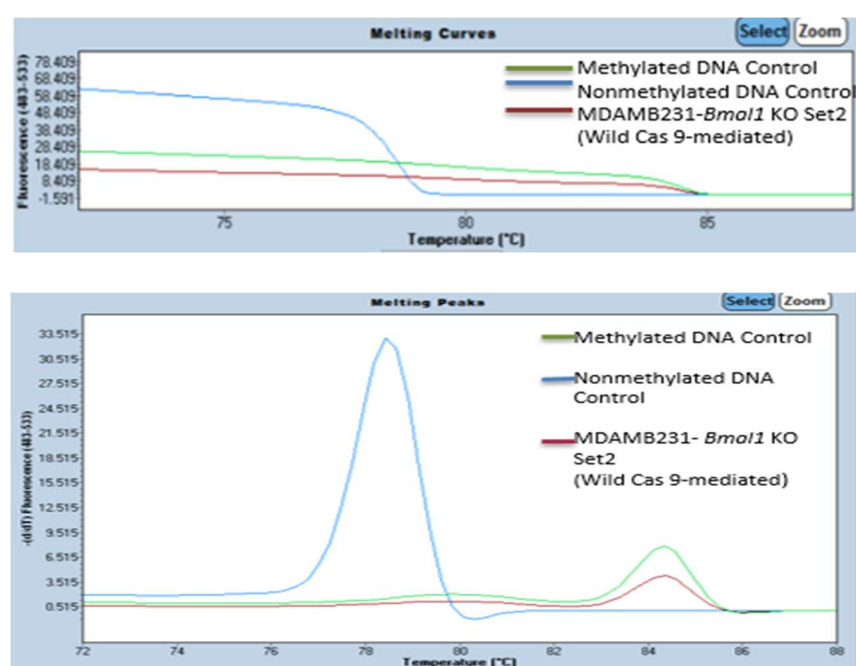


Figure 4.20: Continued

e)



f)

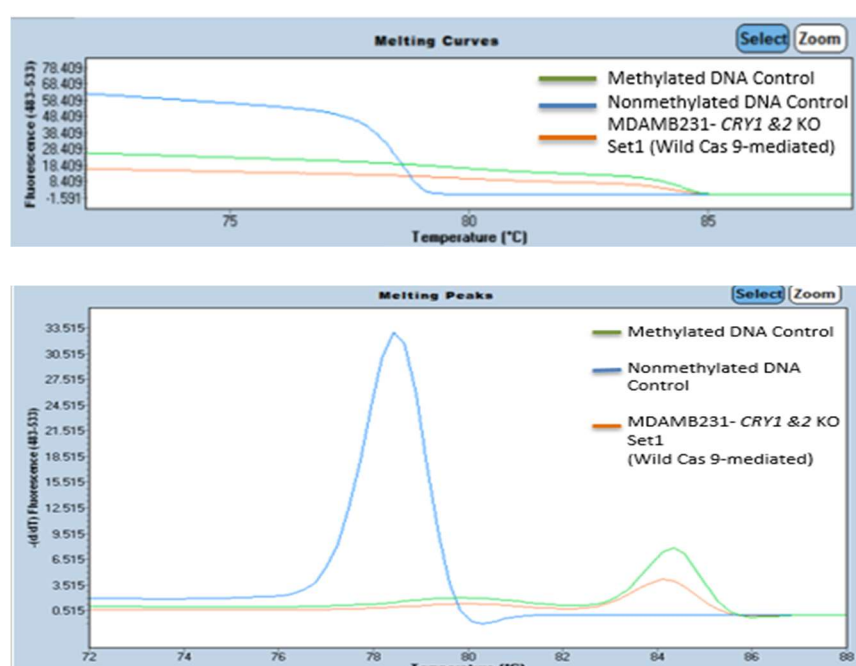


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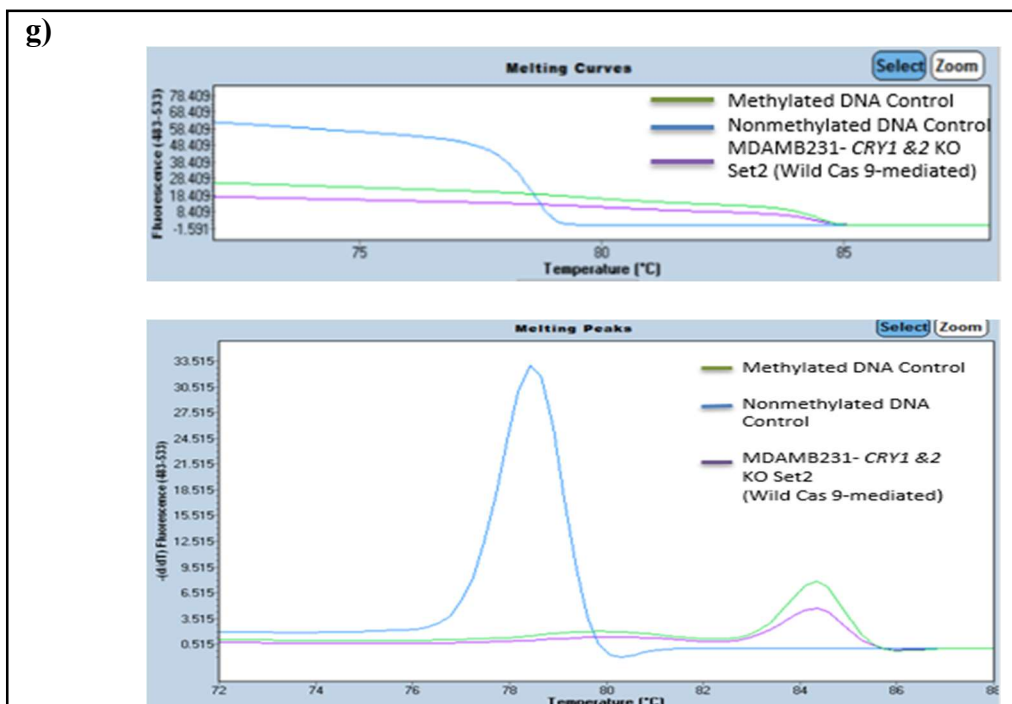


Figure 4.20: Continued

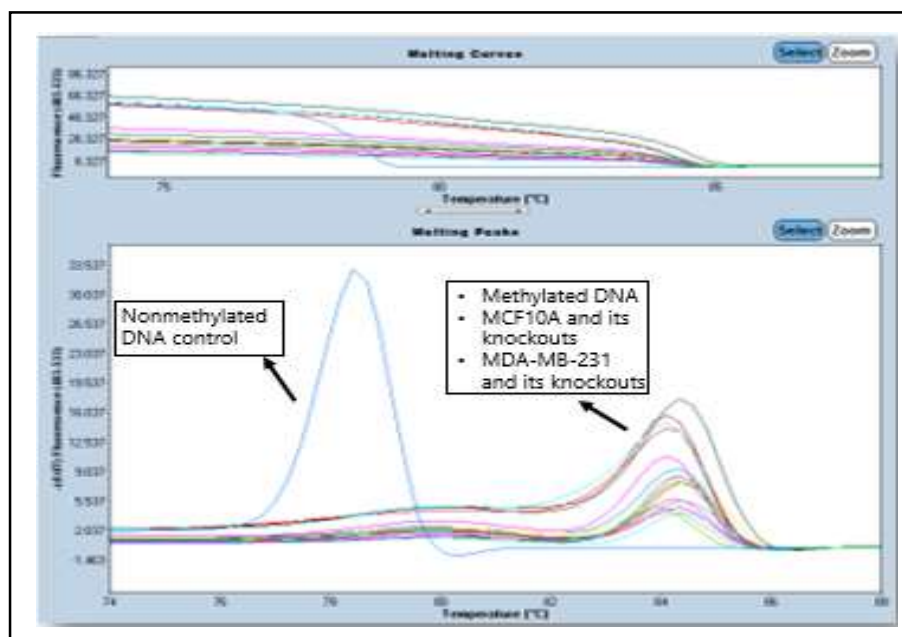


Figure 4.21: RASSF1A melting curves and melting peaks of both MCF10A and MDA-MB-231 cell lines and their knockouts.

Figure 4.18.a shows the MCF10A cell line has shown a non-methylated profile because it displayed same melting curve and melting peaks with nonmethylated DNA control. The MCF10A cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.18.b–e). Figures 4.19.a and 4.20.a shows the MDA-MB-231 cell line has shown a non-methylated profile because it displayed same melting curve and melting peaks with nonmethylated DNA control. The MDA-MB-231 cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.19.b–e and 20.b–g). Figure 4.21 showed that all samples including methylated and non-methylated DNA controlled the *RASSF1A* gene methylation profile.

4.5.3. High Resolution Melting (HRM) Analysis of *RARβ* gene

The results of the HRM analysis with *RARβ* primers show that all samples gave specific HRM curves. The MCF10A cell line and knockouts of the MCF10A cell lines were studied with *RARβ* primers and the obtained HRM profiles are as given in Figure 4.22.

Similarly, MDAMB231 cell lines and their CRISPR knockouts (both Nickase-mediated and lentivirus-mediated) were studied with *RARβ* primers and the obtained HRM profiles are given in Figures 4.23, 4.24, 4.25 and 4.26.

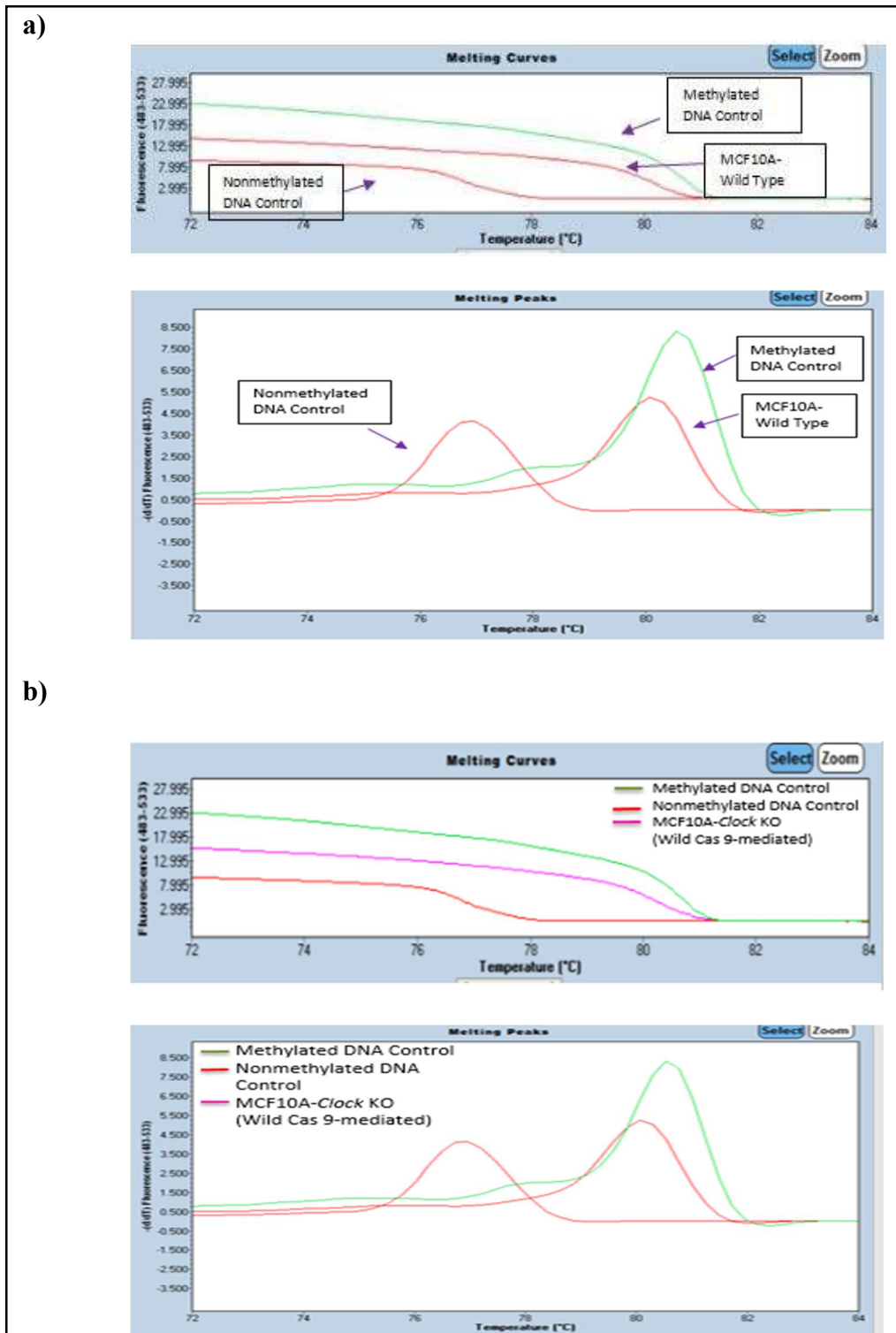
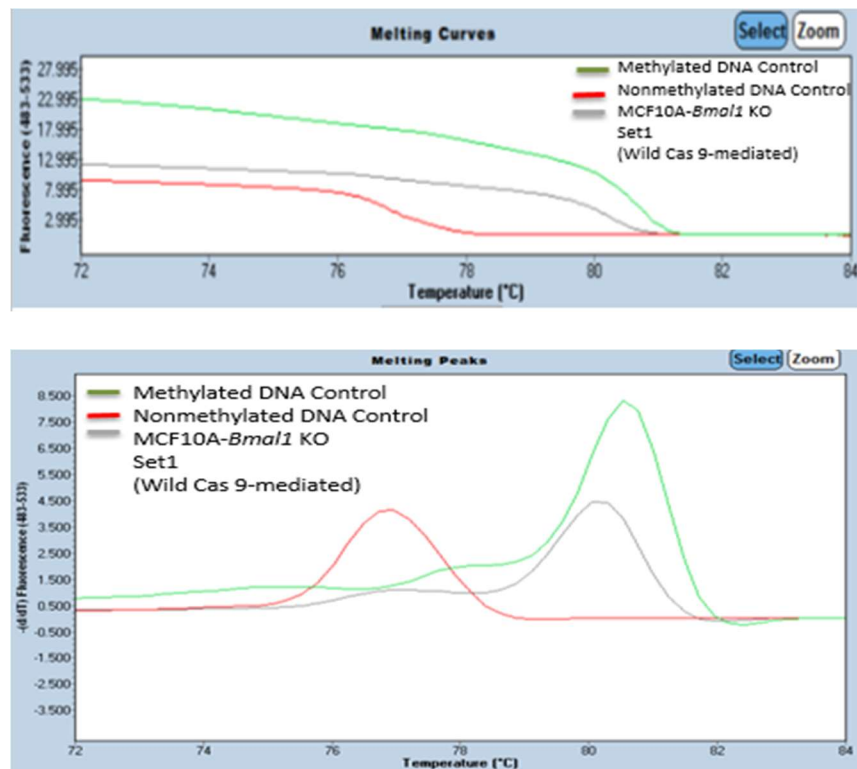


Figure 4.22: RAR β melting curves and melting peaks of MCF10A cell line and its knockouts with Wild-type Cas9. a) MCF10A wild-type cell line HRM profile. b) MCF10A *Clock* knockout cell line HRM profile. c-d) MCF10A *Bmal1* knockout cell line Set1 and Set2 HRM profile, respectively. e) MCF10A *CRY1&2* knockout cell line HRM profile. f) Negative control.

c)



d)

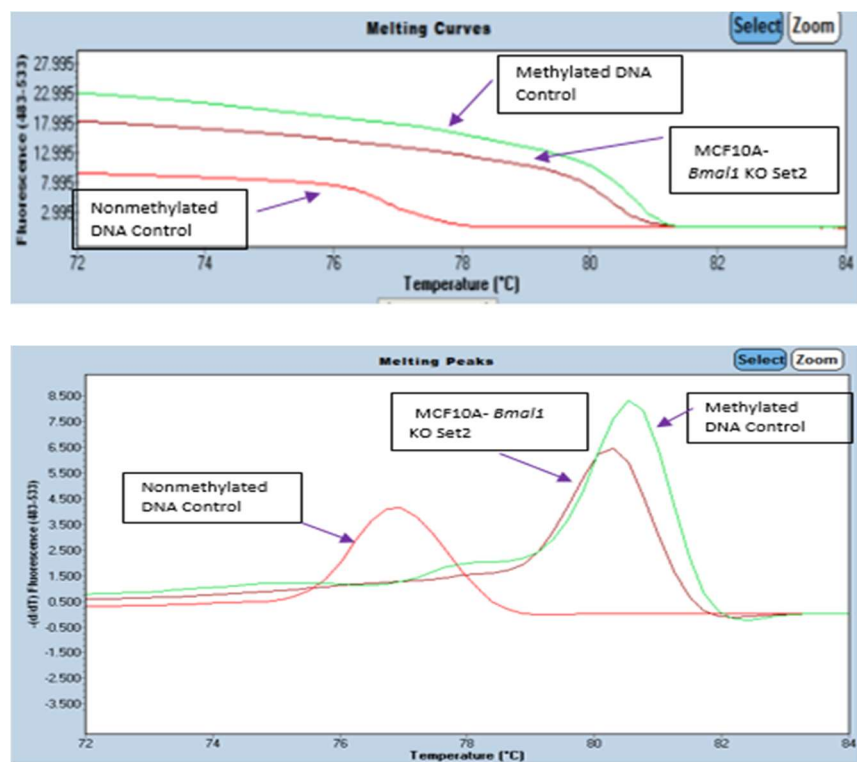
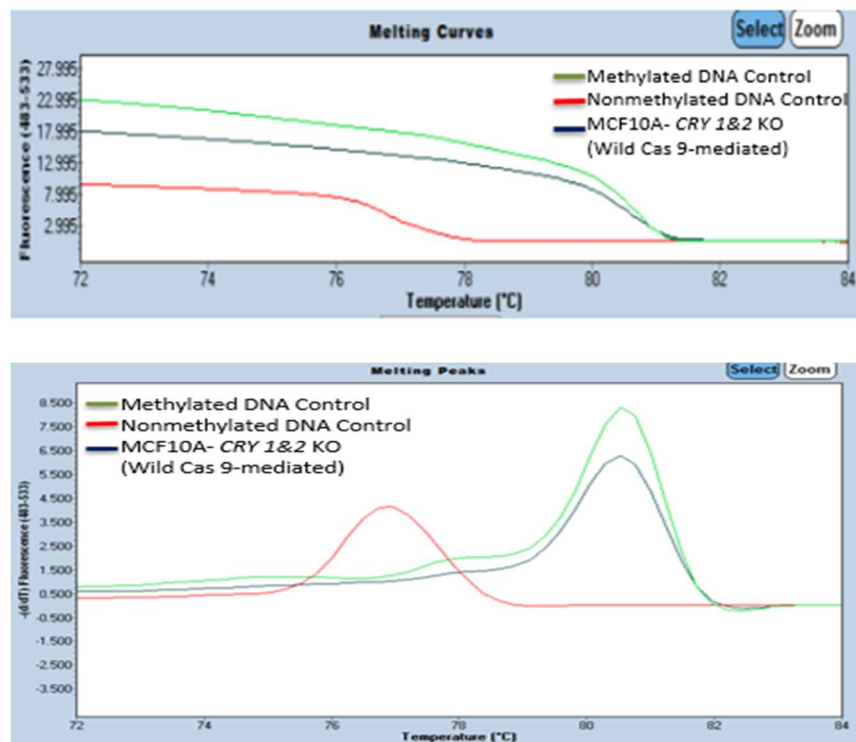


Figure 4.22: Continued

e)



f)

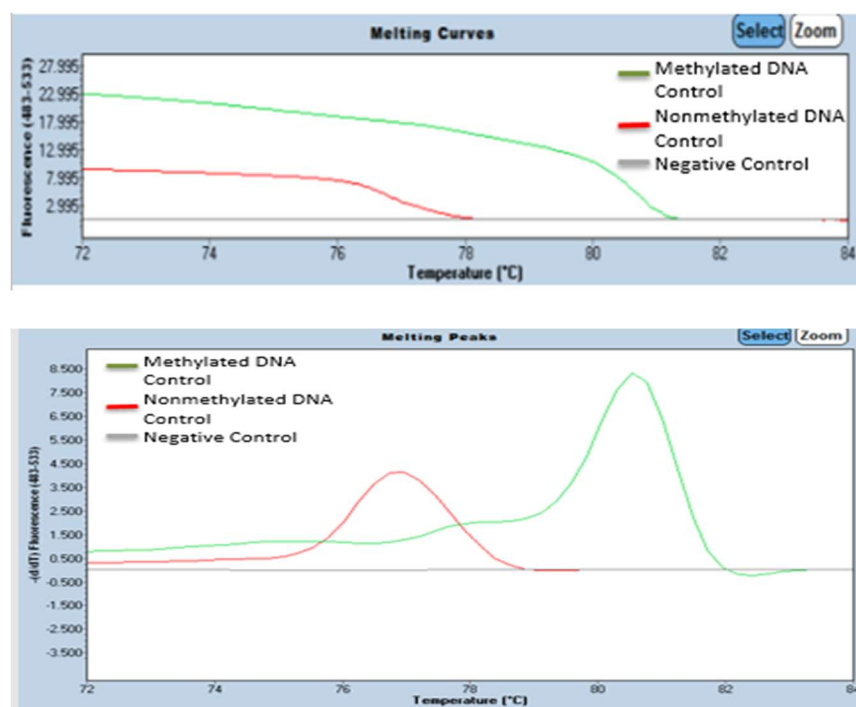


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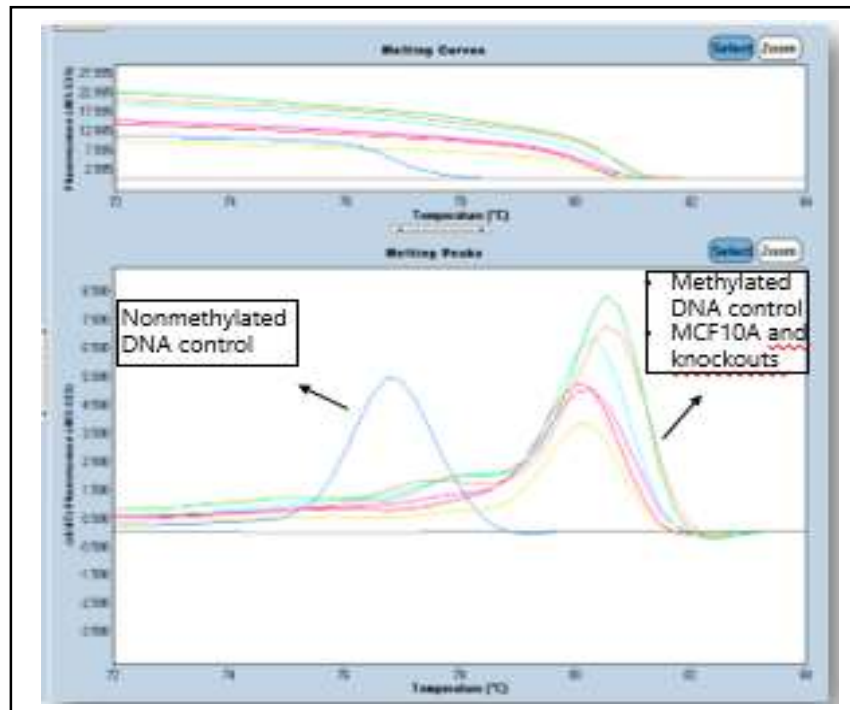


Figure 4.23: RAR β melting curves and melting peaks of the MCF10A cell line and its knockouts.

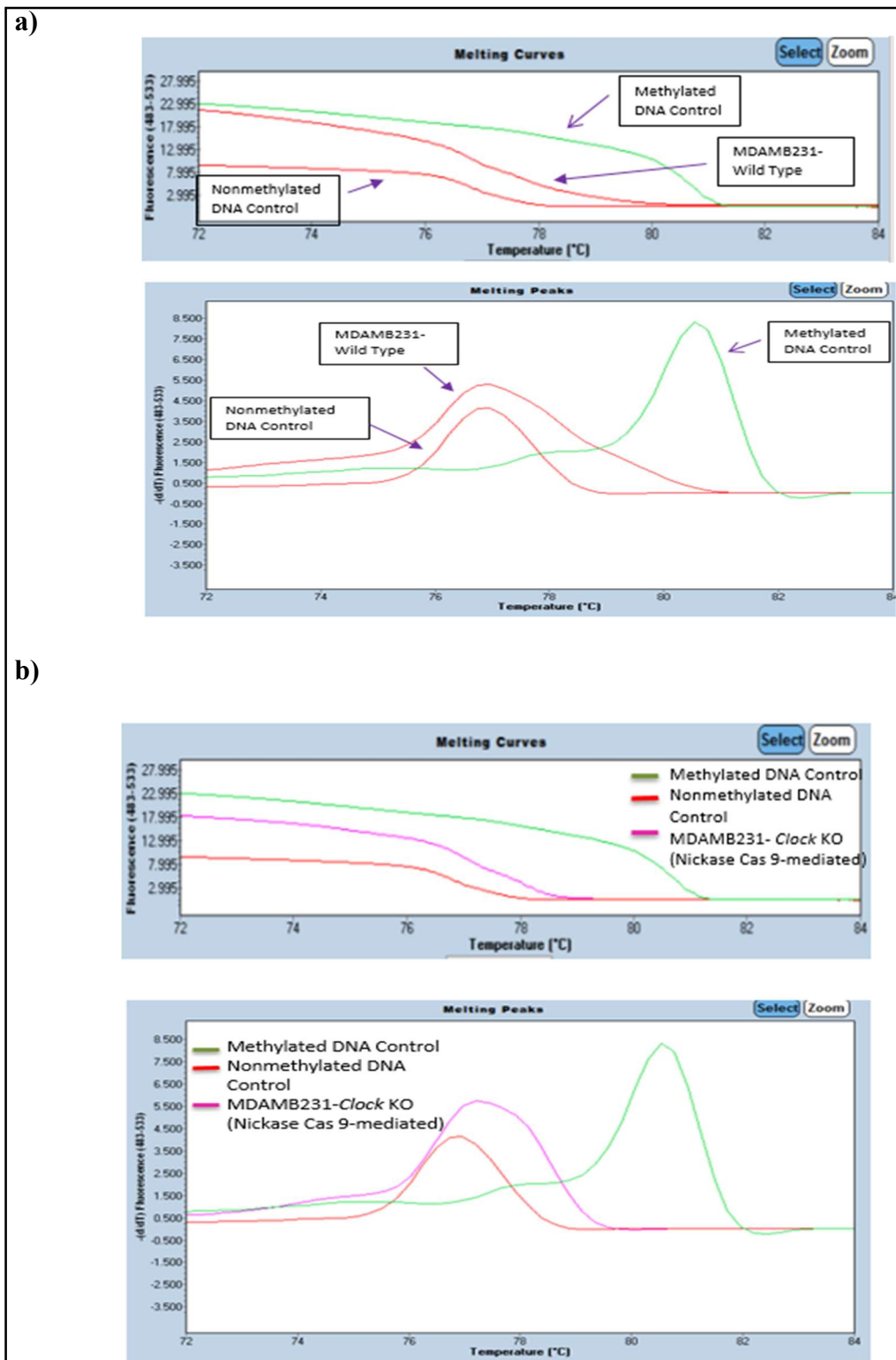
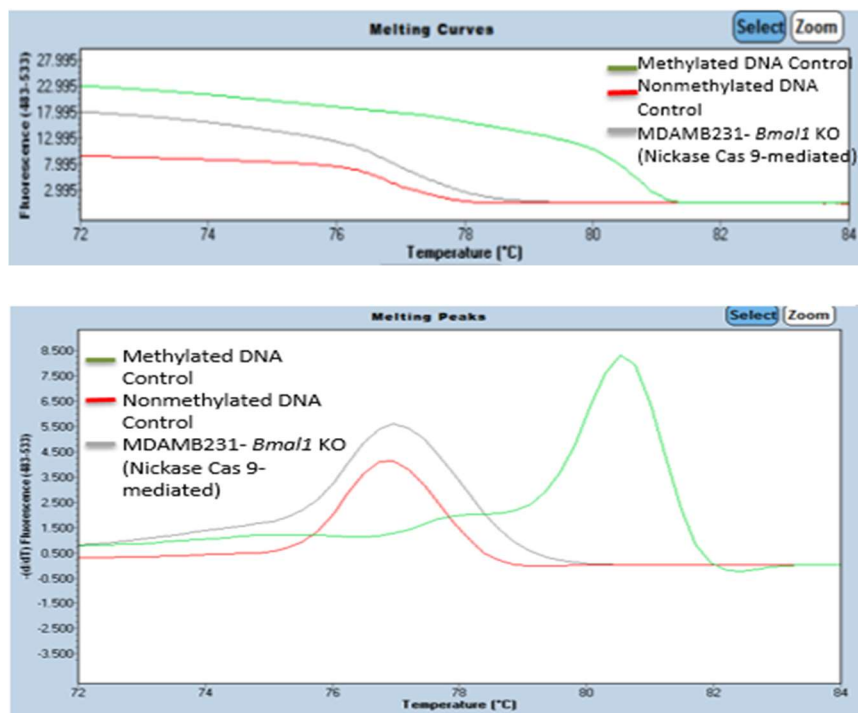


Figure 4.24: RAR β melting curves and melting peaks of the MDA-MB-231 cell line and its knockouts with Nickase Cas9. a) MDA-MB-231 wild-type cell line HRM profile. b) MDA-MB-231 *Clock* knockout cell line (Nickase Cas9) HRM profile. c) MDA-MB-231 *Bmal1* knockout cell line (Nickase Cas9) HRM profile. d) MDA-MB-231 *CRY 1&2* knockout cell line (Nickase Cas9) HRM profile.

c)



d)

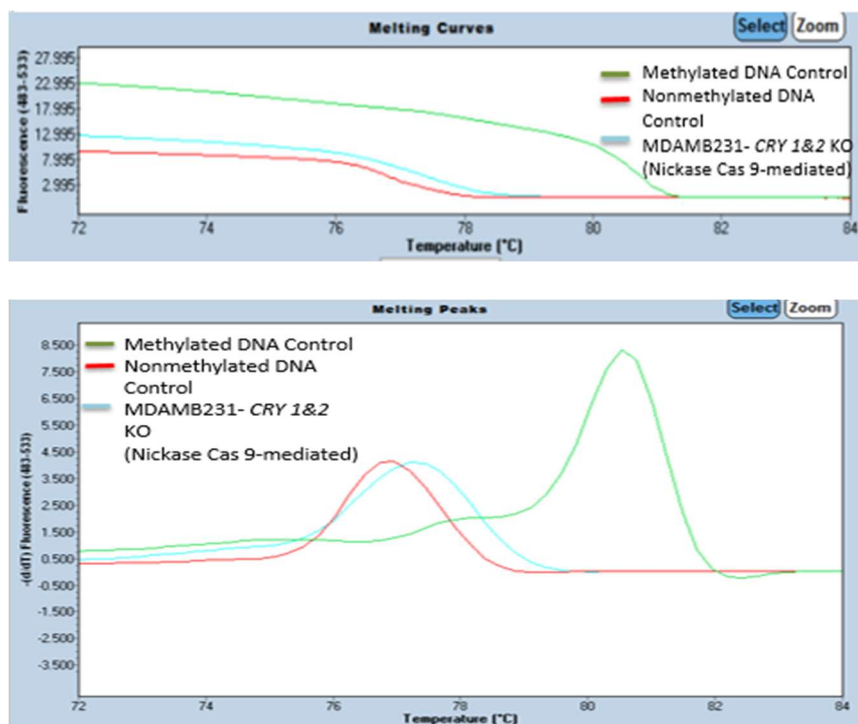


Figure 4.24: Continued

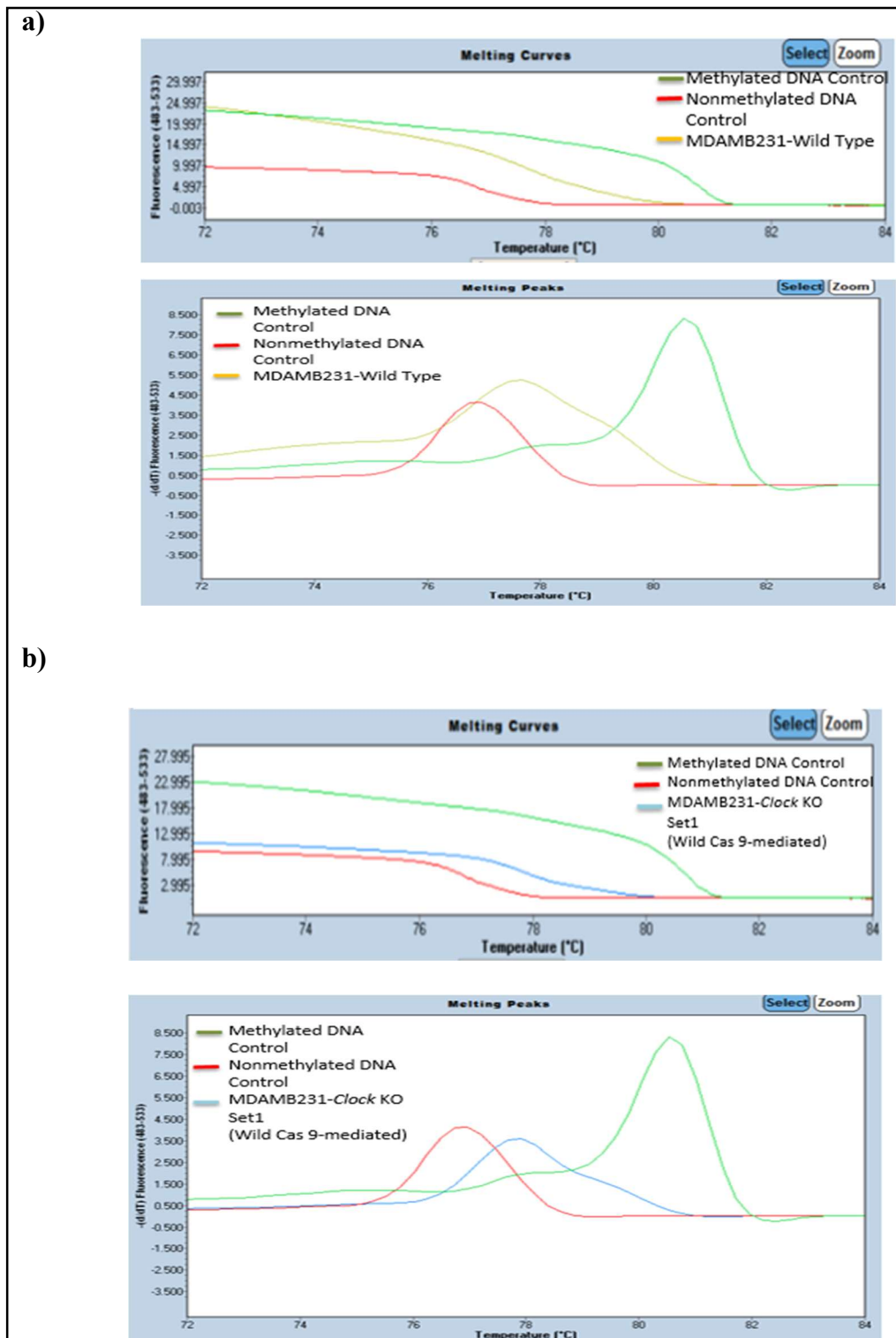
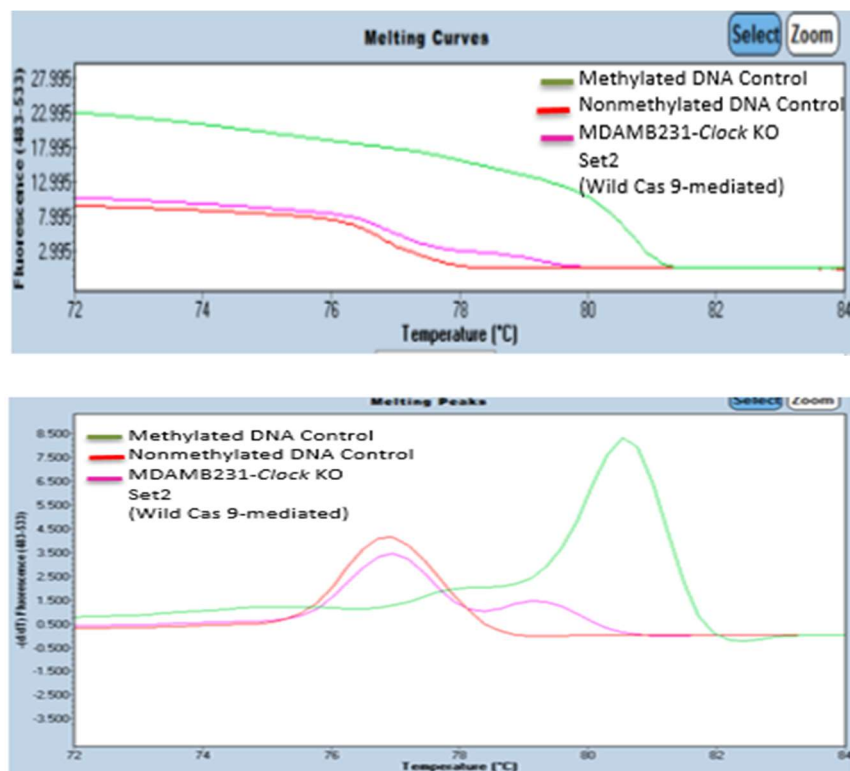


Figure 4.25: RAR β melting curves and melting peaks of the MDA-MB-231 cell line and its knockouts with Wild-type Cas9. a) MDA-MB-231 wild-type cell line HRM profile. b-c) MDA-MB-231 *Clock* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively. d-e) MDA-MB-231 *Bmal1* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively. f-g) MDA-MB-231 *CRY 1&2* knockout cell line (Wild-type Cas9) Set1 and Set2 HRM profile, respectively.

c)



d)

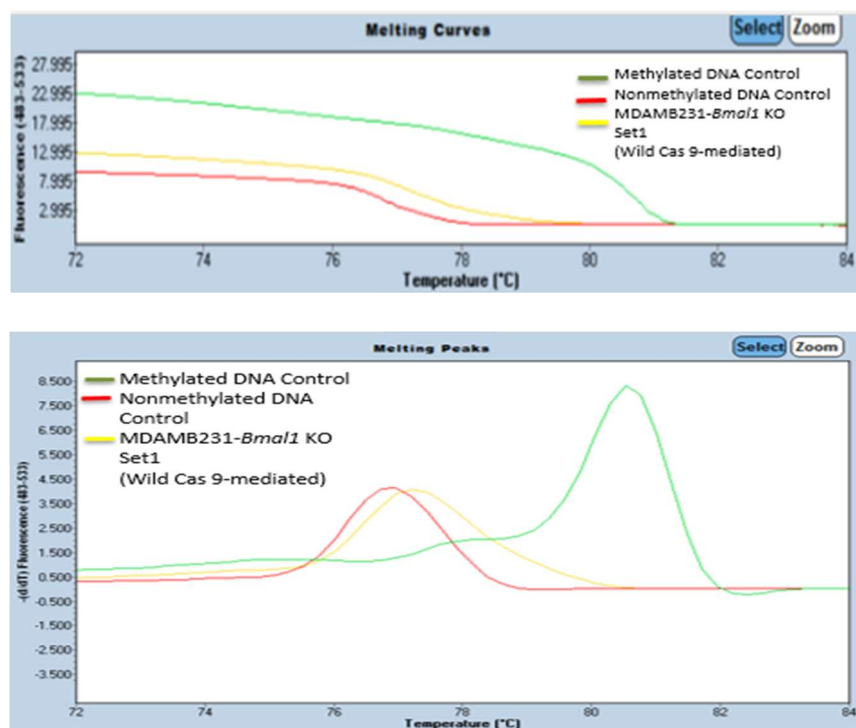
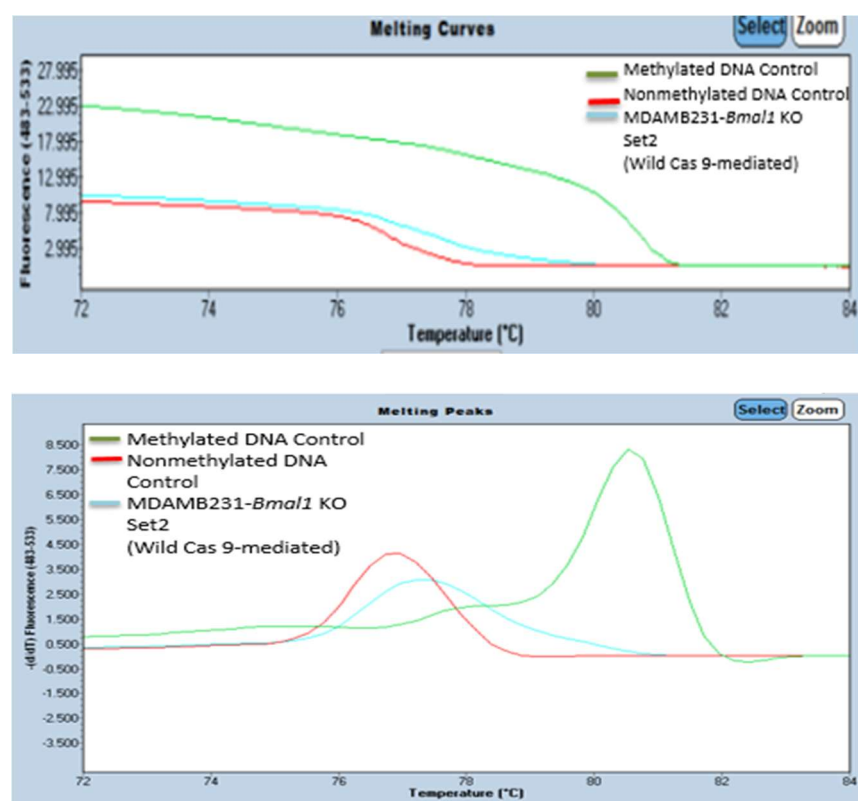


Figure 4.25: Continued

e)



f)

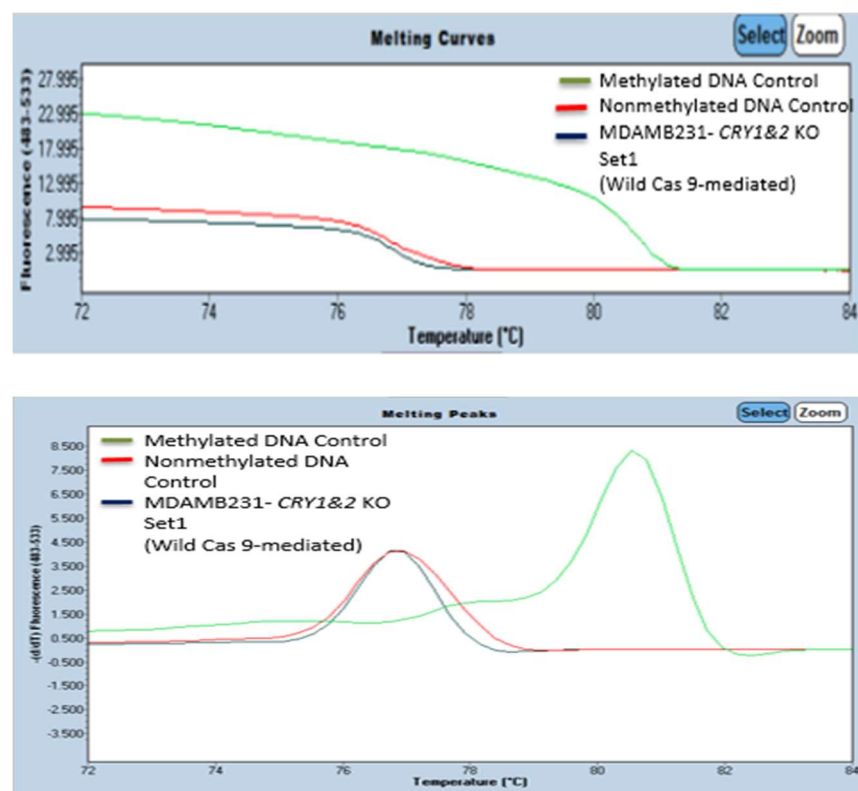


Figure 4.25: Continued

g)

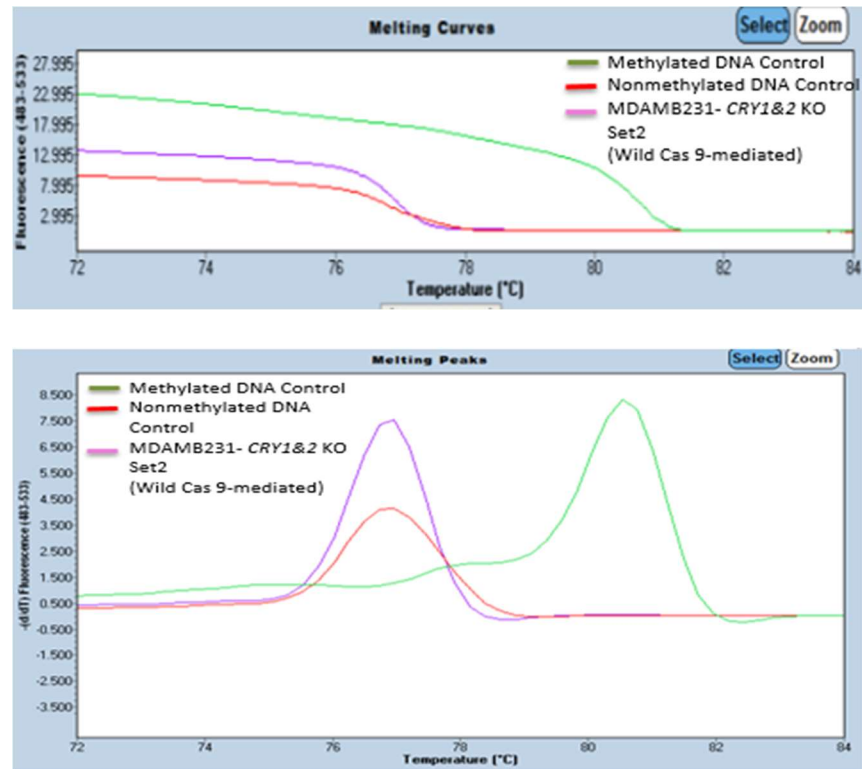


Figure 4.25: Continued

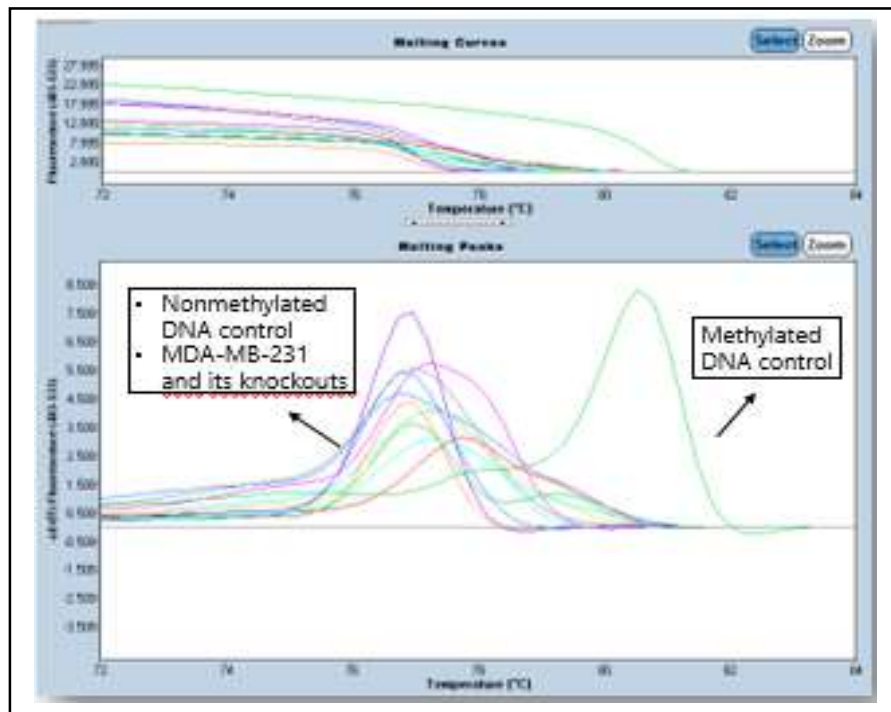


Figure 4.26: RAR β melting curves and melting peaks of MDA-MB-231 cell line and its knockouts.

Figure 4.22a shows the MCF10A cell line has shown a methylated profile because it displayed same melting curve and melting peaks with methylated DNA control. The MCF10A cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.22.b–e). Figure 4.23 showed that MCF10A and its knockouts including methylated and non-methylated DNA controlled the *RAR β* gene methylation profile. Figures 4.24.a and 4.25 shows the MDA-MB-231 cell line has shown a non-methylated profile because it displayed same melting curve and melting peaks with nonmethylated DNA control. The MDA-MB-231 cell line's *Clock*, *Bmal1* and *CRY 1&2* knockouts exhibited same melting curve and melting peaks with own wild type (Figures 4.24.b–e and 25b–g). Figure 4.26 showed that MDA-MB-231 and its knockouts including methylated and non-methylated DNA controlled the *RAR β* gene methylation profile.

4.6. Agarose Gel Electrophoresis of PCR Products

All samples were run on a 2% agarose gel after qPCR. Figure 4.27 have shown that BRCA1 primers do not bind to DNA non-specifically. Similarly, figure 4.28 have shown that RASSF1A primers do not bind to DNA non-specifically. Finally, figure 4.29 have shown that *RAR β* primers do not bind to DNA non-specifically.

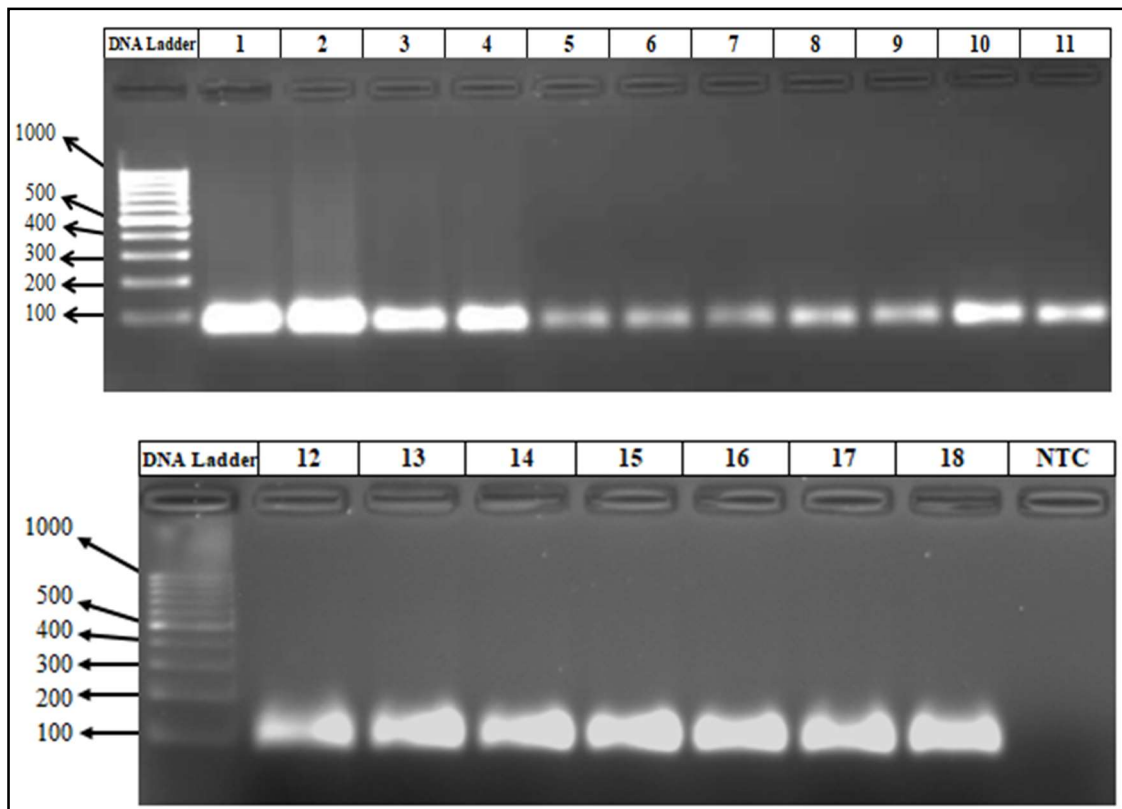


Figure 4.27: The PCR products of the *BRCA1* gene. Amplicons were analysed in 2% DNA agarose gel using gel electrophoresis. GeneRuler 100 bp SM024 (Fermentas) was used as a DNA Ladder. Lane 1: Non-methylated DNA control; lane 2: Methylated DNA control; lane 3: MCF10A-Wild-type; lane 4: MCF10A *Clock* KO; lanes 5 and 6: MCF10A- *Bmal1* KO Set1 and Set 2; lane 7: MCF10A- *CRY 1&2* KO; lane 8: MDA-MB-231- Wild-type; lane 9: MDA-MB-231- *Clock* KO (Nickase Cas9-mediated); lane 10: MDA-MB-231- *Bmal1* KO (Nickase Cas9-mediated); lane 11: MDA-MB-231-*CRY 1&2* KO (Nickase Cas9-mediated); lane 12: MDA-MB-231- Wild-type; lanes 13 and 14: MDA-MB-231- *Clock* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 15 and 16: MDA-MB-231- *Bmal1* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 17 and 18: MDA-MB-231- *CRY 1&2* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; NTC: non-template control.

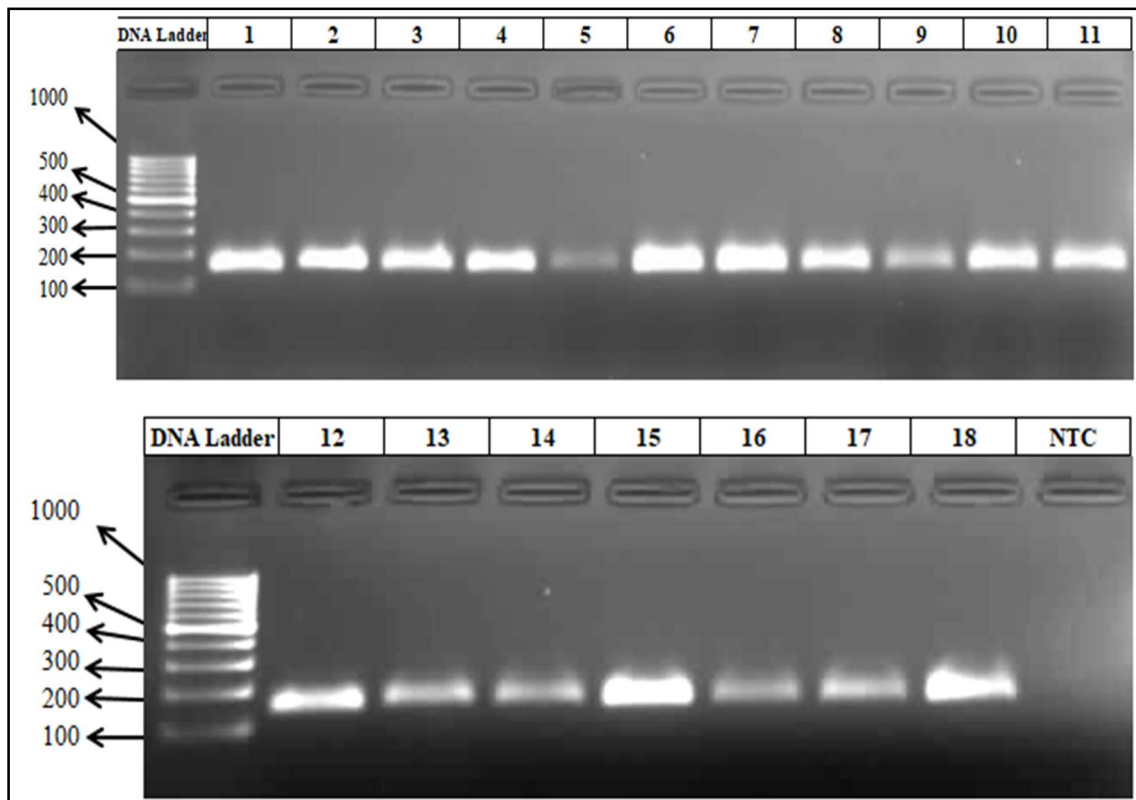


Figure 4.28: The PCR products of the *RASSF1A* gene. Amplicons were analysed in 2% DNA agarose gel using gel electrophoresis. GeneRuler 100 bp SM024 (Fermentas) was used as a DNA Ladder. Lane 1: Non-methylated DNA control; lane 2: Methylated DNA control; lane 3: MCF10A-Wild-type; lane 4: MCF10A *Clock* KO; lanes 5 and 6: MCF10A- *Bmal1* KO Set1 and Set 2; lane 7: MCF10A- *CRY 1&2* KO; lane 8: MDA-MB-231- Wild-type; lane 9: MDA-MB-231- *Clock* KO (Nickase Cas9-mediated); lane 10: MDA-MB-231- *Bmal1* KO (Nickase Cas9-mediated); lane 11: MDA-MB-231-*CRY 1&2* KO (Nickase Cas9-mediated); lane 12: MDA-MB-231- Wild-type; lanes 13 and 14: MDA-MB-231- *Clock* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 15 and 16: MDA-MB-231- *Bmal1* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 17 and 18: MDA-MB-231- *CRY 1&2* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; NTC: non-template control.

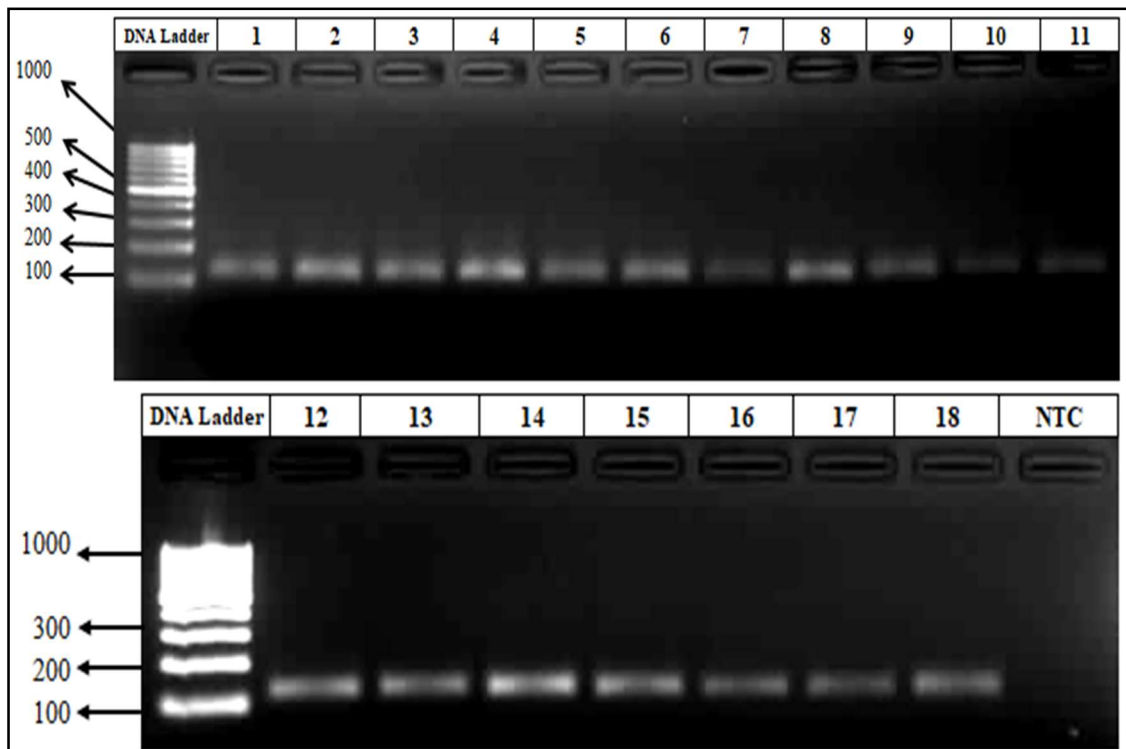


Figure 4.29: The PCR products of *RARβ* gene. Amplicons were analysed in 2% DNA agarose gel using gel electrophoresis. GeneRuler 100 bp SM024 (Fermentas) was used as a DNA Ladder. Lane 1: Non-methylated DNA control; lane 2: Methylated DNA control; lane 3: MCF10A-Wild-type; lane 4: MCF10A *Clock* KO; lanes 5 and 6: MCF10A- *Bmal1* KO Set1 and Set 2; lane 7: MCF10A- *CRY 1&2* KO; lane 8: MDA-MB-231- Wild-type; lane 9: MDA-MB-231- *Clock* KO (Nickase Cas9-mediated); lane 10: MDA-MB-231- *Bmal1* KO (Nickase Cas9-mediated); lane 11: MDA-MB-231-*CRY 1&2* KO (Nickase Cas9-mediated); lane 12: MDA-MB-231- Wild-type; lanes 13 and 14: MDA-MB-231- *Clock* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 15 and 16: MDA-MB-231- *Bmal1* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; lanes 17 and 18: MDA-MB-231- *CRY 1&2* KO Set1 and Set2 (Wild-type Cas9-mediated), respectively; NTC: non-template control.

5. DISCUSSION

Circadian clocks are biological process which occurs with a periodicity of approximately 24 h in many organisms. A lot of molecular, biochemical and behavioural processes are controlled by circadian clock. Disruption of circadian clock affects these biological systems and cause many diseases such as cancer. However, DNA methylation has an important role in the regulation of gene expression, differentiation and development in cancer. Aberrant methylation is observed in many cancer types and may cause loss of differentiation and regulation. Our results have shown relationship between disruption of circadian clock genes, *Clock*, *Bmal1* and *Cry1&2*, on DNA methylation.

In our study, DNAs were isolated from MCF10A and MDA-MB-231 cell lines and their knockout clones of *Clock*, *Bmal1* and *Cry1&2* genes. After bisulfide treatment of the DNA samples, different HRM dyes were studied for detect the best HRM dye to analyse methylation profile. All master mixes showed a shoulder structure, except ZymoTaq™ qPCR Premix (Figure 4.11). This shoulder structure was not a non-specific binding as shown in Figure 4.12. Then, the Human Methylated and Non-methylated DNA Set was studied with DAPK1 primers and its showed that the accuracy of bisulfide treatment in Figure 4.13. All samples were subjected to HRM analysis after qPCR, and results were demonstrated as in Figures 4.14–4.26.

The *BRCA1* gene mutation is generally known to be associated with breast cancer. The BRCA1 protein plays a significant role in DNA double-strand-break repair [Roy, Chun, and Powell, 2016]. In addition, the *BRCA1* gene can undergo epigenetic inactivation in sporadic breast cancer via DNA methylation in its promoter-associated CpG island [Stefansson et al., 2012]; [Bosviel et al., 2012]. On this basis, *BRCA1* gene methylation analysis was studied in MCF10A and MDAMB-231 cell lines and their knockouts. The MCF10A cell line has shown a non-methylated profile (Figure 4.14a). We performed the knockout of *Clock*, *Bmal1* and *CRY 1&2*, and investigated the effect of disrupting the circadian rhythm on non-tumorigenic epithelial breast cell line. All demonstrated a non-methylated profile of MCF10A (Figures 4.14.b–e). Similarly, the MDA-MB-231 cell line showed a non-methylation profile (Figures 4.15.a and 4.17.a); this result is as expected [Yang et al., 2001]. We performed the knockout of *Clock*, *Bmal1* and *CRY 1&2* by the Nickase Cas9 CRISPR method and Wild-type Cas9

CRISPR method for the MDA-MB-231 cell line. We investigated the effect of disrupting the circadian rhythm on the tumorigenic epithelial breast cell line. All demonstrated a non-methylated profile of MDA-MB-231 (Figures 4.15.b–e and 4.16b–g). Figure 4.17 showed that all samples including methylated and non-methylated DNA controlled the *BRCA1* gene methylation profile.

BRCA1 has a role in DNA repair, transcription, G2/M cell cycle checkpoint regulation and spindle checkpoint. Therefore, the disruption of the circadian clock genes does not affect these pathways by changing BRCA1 methylation.

RASSF1A is a tumour suppressor gene and plays a role in the loss of negative regulation control of cell proliferation through the inhibition of G1/S- phase progression in tumour development. It is commonly methylated in human breast cancer [Das and Singal, 2004]. On this basis, *RASSF1A* gene methylation analysis was studied in both MCF10A and MDAMB-231 cell lines and their knockouts by Nickase Cas9 and Wild type Cas9. The MCF10A cell line has shown a methylated profile (Figure 4.18.a). We performed knockout of *Clock*, *Bmal1* and *CRY 1&2* and investigated the effects of disrupting the circadian rhythm on non-tumorigenic epithelial breast cell line. All have shown a methylated profile of MCF10A knockouts (Figures 4.18.b–e). Similarly, the MDA-MB-231 cell line has shown a methylated profile (Figures 4.19.a and 4.20.a); this result is as expected [Dammann et al., 2001]. We performed knockout of *Clock*, *Bmal1* and *CRY 1&2* by the Nickase Cas9 CRISPR method and Wild-type Cas9 CRISPR method with the MDA-MB-231 cell line. We investigated the effect of disruption circadian rhythm on tumorigenic epithelial breast cell line. All of them demonstrated a methylated profile of MDA-MB-231 (Figures 4.19.b–e and 4.20.b–g). Figure 4.21 shows that all samples including methylated and non-methylated DNA control the *RASSF1A* gene methylation profile.

RASSF1A is associated with cell cycle regulation, microtubule network and apoptosis through the mitogen-activated protein (MAP) kinase pathway. Therefore, it can be said that the disrupting the circadian clock genes does not affect these pathways by changing the *RASSF1A* methylation.

Lastly, *RARβ* gene methylation profile was investigated. *RARβ* is a tumour suppressor gene and commonly aberrant methylation in breast cancer [Hoque et al., 2006], and *RARβ* gene methylation analysis was studied in MCF10A and MDAMB-231 cell lines and these knockouts. The MCF10A cell line showed a methylated profile (Figure 4.22.a) and this result is expected [Batoon et al., 2018]. We performed the

knockout of *Clock*, *Bmal1* and *CRY 1&2*, and investigated the effect of circadian rhythm disruption on the non-tumorigenic epithelial breast cell line. All showed a methylated profile for MCF10A knockouts (Figures 4.22.b–e). Similarly, the MDA-MB-231 cell line showed a non-methylated profile (Figures 4.24.a and 4.25.a). We performed a knockout of *Clock*, *Bmal1* and *CRY 1&2* by the Nickase Cas9 CRISPR method and Wild-type Cas9 CRISPR method with MDA-MB-231 cell line. We investigated the effect of circadian rhythm disruption on the tumorigenic epithelial breast cell line. All demonstrated a non-methylated profile for MDA-MB-231 (Figures 4.24.b–e and 4.25.b–g). Figures 4.23 and 4.26 showed that all samples including methylated and non-methylated DNA control the *RARβ* gene methylation profile.

RARβ can regulate its own expression and that of its downstream genes. This function generally results in the inhibition of cell growth. Therefore, the disruption of circadian clock genes does not affect these pathways by changing *RARβ* methylation.

In summary, the knockout of *Clock*, *Bmal1* and *CRY 1&2* genes has been found not to affect the methylation status of *BRCA1*, *RASSF1A* and *RARβ* genes that are associated with breast cancer. This study was just qualitative analysis. Our data shows the 100% methylation status or non-methylation status of genes. In the future, it could study quantitative analysis by DNA sequencing, chip-based digital PCR, droplet digital PCR or a standard DNA methylation measurement system. Specifically, we observed greater deviation in the *RARβ* methylation profile than in other genes. Analysing the percentage to which this gene was methylated, reveals whether the deviation is associated with little methylation. Finally, working with the assistance of more gene panels helps understand how the circadian clock disruption on DNA methylation is related to cancer.

A study was undertaken in which a methylation check at the genomic level was planned, with the exception of the limited genes examined in this study. Populations in both phases quench each other because the biological clock used in wild-type cells was not reset. The difference between wild type cell lines and its knockout clones may not be distinguished because this creates the average knockout phenotype in the wild type cells.

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BIOGRAPHY

Meltem AŞICIOĞLU was born in Kusadası, Turkey, August 1, 1993. She received her B.Sc. in Molecular Biology and Genetics (100% full scholarship) at the Department at Istanbul Yeni Yuzyıl University in 2015. She joined the M.Sc. Program in Gebze Technical University, Graduate School of Natural and Applied Science, Molecular Biology and Genetics Department and then she graduated with an MSc in 2018. She joined the South Korea–Turkey project in the Scientific and Technological Research Council of Turkey National Metrology Institute (TUBITAK UME) Bioanalysis Laboratory group. She worked for two years under the supervision of Assoc. Prof. Dr. Nuri OZTURK and co-supervision of Assoc. Prof. Dr. Muslum AKGOZ.

APPENDICES

Appendix A: Human BRCA1 gene, partial cds

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CTGCTGGNCCGGGTGCTAGGNCCCTGACTGCCCGGGGCCGGGGGTGCGG
GGCCCCGCTGAGCCCGCGCCACCTGGAACCTCGCGCTGGCTGGCGAGCGC
TGCGCGCAGNCCCAGTTCCCACACCCGCCTCTCCCTCCACACTTCCCCGC
AAGCAGAGGGAGCCGGCTCTGGCTTCGGCCAGCCCAGAGAGGGGGCCCCC
ACAGCGCAGTGGCGGGCTGAAGGGCTCCTCCAGCACGGNCAGAATGGAC
GCCAAGGNCGAGGAGGCGCCGAGAGCGAGCGAGGGCTGCTAGCACGTT
GTCACCTCGCATTCTGAACCACAGACTCTCCAACCTCTCCGNGCTTTTCG
CCCCTCGGTCCCTCAGAACACGAAGGGCTCTCTCATCCTGTCACTAAAA
CGATTAGCTGTCCGGAGACACGGAAAAAGTCGCCCCCTCTTCTTTGCAGGA
TTCCTCCCTTGAACCTTCTCCAAACCCTCTTAGTGTGACGTGACCCACCCC
TAGCTAACCAGGCTGCTTCCTTACCAGCTTCCCGCCCCCTGGGGAGGCG
GCAATGCAAAGACCGTCCGCTGCCAGCTCTGCCGCTATCTCTGTGGGGTG
AATCTAACATGGCGGACAAAGACAGTAACTAGTCCCGTTTCTCCGCGTTT
TCGCCAAGAAGATTGGCTCTTACCACTTGTCCCTCAAAACGACCACCCCA
TTGACTGGTGGCGATTGCGTCGACGGAGACGGGGGCAAAAGCAAGCTGAA
CCCGAAAAATAACAAACACTGGGGCTGAGGGGTGGAACACGAGTGCGC
AGACATGGGCCAGAGCGCATTTCCCCTGCCCCAGGCAAATTCGGCGCTCA
CTGCGTCCCCGCAGGCCACTGACCTTACAAGACTACTTGCCCCAGACTCC
TGGGGCTGGATGGGAATTGTAGTCTCCCTAAAGAGTTGTACGTATCTTTT
TAAGGCCTAGTTTCTGCTTTCNAAATACGAAAACATAACACTCCAGTCCA
TAACTGTTGACAAGTACAAGCGCGCACAGGTCTCCAATCTATCCACTGGA
TTTCCGTGAGAATTGTGCCCGCTCTGGTATTGGATGTTCCCTCTCCATAAGA
CTACAGTTTCTAAGGAACACTGTGGCGAAGACCTTTCATTCCGCAACGCA
TGCTGGAAATAATTATTTCCCTCCACCCCCCAACAATCCTTATTACTTAT
ATTTACCGAAACTGGAGACCTCCATTAGGGCGGAAAGAGTGGGGGATTG
GGACCTCTTCTTACGACTGCTTTGGACAATAGGTAGCGATTCTGACCTTC
GTACAGCAATTACTGTGATGCAATAAGCCGCAACTGGAAGAGTAGAGGC
TAGAGGGCAGGCACTTTATGGCAAACCTCAGGTAGAATTCTTCCTCTTCCG
TCTCTTTCCTTTTACGTCATCCGGGGGCAGACTGGGTGGCCAATCCAGAG
CCCCGAGAGACGCTTGGCTCTTTCTGTCCCTCCCATCCTCTGATTGTACCT
TGATTTTCGTATTCTGAGAGGCTGCTGCTTAGCGGTAGCCCCCTTGGTTTCCG
TGGCAACGGAAAAGCGCGGGAATTACAGATAAATTAAAACCTGCGACTGC
GCGGCGTGAGCTCGCTGAGACTTCCTGGACGGGGGACAGGCTGTGGGGT
TTCTCAGATAACTGGGCCCCCTGCGCTCAGGAGGCCTTCACCCTCTGCTCT
GGGTAAAGGTAGTAGAGTCCCGGGAAAGGGACAGGGGGGCCAAGTGAT
GCTCTGGGGTACTGGCGTGGGAGAGTGGATTTCCGAAGCTGACAGATGG
GTATTCTTTGACGGGGGGTAGGGGCGGAACCTGAGAGGCGTAAGGCGTT
GTGAACCCTGGGGAGGGGGGAGTTTGTAGGTCGCGAGGGAAGCGCTGA
GGATCAGGAAGGGGGCACTGAGTGTCCGTGGGGGAATCCTCGTGATAGG
AACTGGAATATGCCTTGAGGGGGACACTATGTCTTTAAAACGTCGGCTG
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Figure A1.1: Human BRCA1 original gene (GenBank: U37574.1)

GTCATGAGGTCAGGAGTTCCAGACCAGCCTGACCAACGTNGGTGAAACT
 CCGTCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGCCGCTCCAGC
 TACTCAGGAGGCTGAGGCAGGAGAATCGCTAGAACCCGGGAGGCCGAG
 GTTGCAGTGAGCCGAGATCGCGCCATTGCACTCCAGCCTGGGCGACAGA
 GCGAGACTGTCTCAAAACAAAACAAAACAAAACAAAACAAAACACC
 GGCTGGTATGTATGAGAGGATGGGACCTTGTGGAAGAAGAGGTGCCAGG
 AATATGTCTGGGAAGGGGAGGAGACAGGATTTTGTGGGAGGGAGAACTT
 AAGAAGTGGATCCATTTGCGCCATTGAGAAAGCGCAAGAGGGGAAGTAGA
 GGAGCGTCAGTAGTAACAGATGCTGCCGGCAGGGATGTGCTTGAGGAGG
 ATCCAGAGATGAGAGCAGGTCACTGGGAAAGGTTAGGGGCGGGGAGGC
 CTTGATTGGTGTGTTGGTTTGGTCGTTGTTGATTTTGGTTTTATGCAAGGGAA
 AGAAAACAACCAGAAACATTGGAGAAAGCTAAGGCTACCACCACCTACC
 CGGTCAGTCACTCCTCTGTAGCTTTCTCTTTCTTGAGAAAGGAAAAGAC
 CCAAGGGGTTGGCAGCAATATGTGAAAAAATTCAGAATTTATGTTGTCTA
 ATTACAAAAGCAACTTCTAGAATCTTTAAAAATATAGGACGTTGTCATT
 AGTTCTTTGGTTTGTATTATTCTAAAACCTTCCAAATCTTAAATTTACTTT
 ATTTTAAATGATAAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGA
 TATATATATATGTTTTTCTAATGTGTTAAAGTTCATTGGAACAGAAAGAA
 ATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAATGTCATTAATGC
 TATGCAGAAAATCTTAGAGTGTCCCATCTGGTAAGTCAGCACAAAGAGTGT
 ATTAATTTGGGATTCCTATGATTATCTCCTATGCAAATGAACAGAATTGA
 CCTTACATACTAGGGAAGAAAAGACATGTCTAGTAAGATTAGGCTATTGT
 AATTGCTGATTTTCTTAACTGAAGAAGTTTAAAAATATAGAAAATGATTC
 CTTGTTCTCCATCCACTCTGCCTCTCCCACTCCTCTCCTTTTCAACACAAA
 TCCTGTGGTCCGGGAAAGACAGGGACTCTGTCTTGATTGGTTCTGCACTG
 GGGCAGGAATCTAGTTTAGATTAAGTGGCATTGTTGGCTTTTCTTCCAGCTC
 TAAAACAAGCTCCATCACTTGAAATGGCAAATAAAATCATGGATGAGG
 CCGAGGGCGGTGGCTTATGCCTGTAATCCCAGCACTTTGGGAGGCCAAG
 GTGGTAGGATCACGAGGTCAGGAGATCGAGACCATCCTGGCCAACATGG
 TGAAACCCCTCTCCACTAAAAATACAAAAATTAGCTGGGCGTAGTGGC
 ATGTGCCTGTAATCCCAGCTACTCAGGAGGCTGAGGCAGGAGAATCACTT
 GAACCAGGAGGCAGATGTTGCTGTGAGCCAATATGGCACCCTGAAGTCA
 CAGCGACAGAGCTAACTCCATCTCAAAAAAAAAAAAAAAAAAAAAANA
 AACATGGATGATCGGTGTCGTTGAGAGGATAGGTATTTGGAAGAACCTTT
 GTTTGAAACTGGCTCTGTACATACAATGAAATTACATACTTATTTACATA
 CAATGAAATGCAGAGGTTTTTTTTTTTATATAGGATCTCTGTGAGAGGCT
 GGAGTGCAGTGGTGCTATCACAGCTCA

Figure A1.1: Continued

GC% of BRCA1 promoter region is given in Figure A.1.2. It was determined by website (<http://canna.cau.ac.kr/util/cgi-web/index.html>).

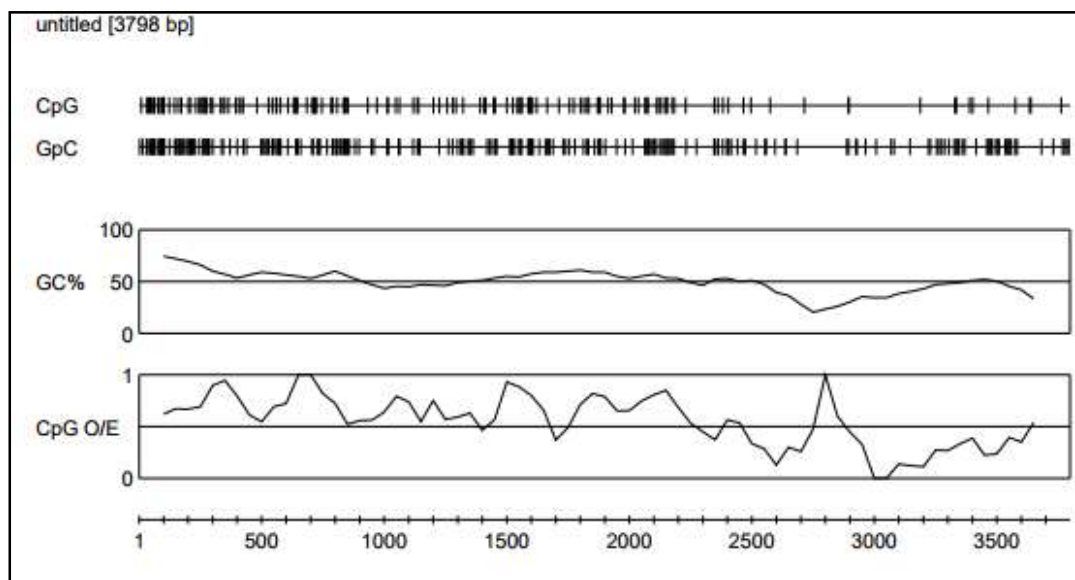


Figure A1.2: GC% of BRCA1 promoter region

Primers details are given in below.

BRCA1-F GGAAAAGCGCGGGAATTATAG
 BRCA1-R ATCCCCCGTCCAAAAAATCTCAAC (Reverse Complement-
 GTTGAGATTTTTTGGACGGGGGAT)

Methylated region of BRCA1 gene is given in Figure A1.3.

TTGTTGGNTCGGGTGTTAGGNTTTTGATTGTTCGGGGTCGGGGGTGCGGG
 GTTCGTTGAGTTCGCGTTTATTTGGAATTCGCGTTGGTTGGCGAGCGTTGC
 GCGTAGNTTTTAGTTTTTATATTCGTTTTTTTTTTATATTTTTTCGTAAGTA
 GAGGGAGTCGGTTTTGGTTTTCGGTTAGTTAGAGAGGGGTTTTATAGCG
 TAGTGCGGGTTGAAGGGTTTTTTTAGTACCGGNTAGAATGGACGTTAAGG
 NCGAGGAGGCGTCGAGAGCGAGCGAGGGTTGTTAGTACGTTGTTATTTC
GTATTTTGAATTATAGATTTTTTAATTTTTTCGNGTTTTTCGTTTATTCGGT
 TTTTTAGAATACGAAGGGTTTTTTTATTTTGTATTAAAACGATTAGTTGT
 TCGGAGATACGGAAAAAGTCGTTTTTTTTTTTTGTAGGATTTTTTTTTTTGA
 ATTTTTTTAAATTTTTTTAGTGTGACGTGATTTTATTTTTAGTTAATTTAGG
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 GTTCGTTGTTAGTTTTGTCGTTATTTTTGTGGGGTGAATTTAATATGGCGG
 ATAAAGATAGTAATTAGTTTCGTTTTTTTCGCGTTTTTCGTTAAGAAGATTGG
 TTTTTATTATTTGTTTTTTAAAACGATTATTTTATTGATTGGTGGCGATTGC
 GTCGACGGAGACGGGGTAAAAGTAAGTTGAATTCGAAAAATAATAAATA
 TTGGGGTTGAGGGGTGAATTACGAGTGCCTAGATATGGGTTAGAGCGT
 ATTTTTTTTGTTTTAGGTAAATTCGGCGTTTATTGCGTTTTTCGTAGGTTATT
 GATTTTATAAGATTATTTGTTTTAGATTTTTGGGGTTGGATGGGAATTGTA
 GTTTTTTTAAAGAGTTGTACGTATTTTTTTAAGGTTTAGTTTTGTTTTTNA
 AATACGAAAATATAATATTTTAGTTTATAATTGTTGATAAGTATAAGCGC
 GTATAGGTTTTTAATTTATTTATTGGATTTTCGTGAGAATTGTGTTCGTTTT
 GGTATTGGATGTTTTTTTTTATAAGATTATAGTTTTTAAGGAATATTGTGG
 CGAAGATTTTTTATTTCTAACGTATGTTGGAAATAATTATTTTTTTTTAT
 TTTTTAATAATTTTTATTATTTATATTTATCGAAATTGGAGATTTTTTATTA
 GGGCGGAAAGAGTGGGGGATTGGGATTTTTTTTTACGATTGTTTTGGATA
 ATAGGTAGCGATTTTGATTTTCGTATAGTAATTATTGTGATGTAATAAGTC
 GTAATTGGAAGAGTAGAGGTTAGAGGGTAGGTATTTTATGGTAAATTTAG
 GTAGAATTTTTTTTTTTTTCGTTTTTTTTTTTTTACGTTATTCGGGGGTAGAT
 TGGGTGGTTAATTTAGAGTTTCGAGAGACGTTTGGTTTTTTTTGTTTTTTTT
 ATTTTTTGATTGTATTTGATTTCTATTTTGAGAGGTTGTTGTTTAGCGGT
 AGTTTTTTGGTTTTCGTGGTAACGGAAAAGCGCGGGAATTATAGATAAAT
 TAAAATTGCGATTGCGCGGCGTGAGTTCGTTGAGATTTTTTGGACGGGGG
 ATAGGTTGTGGGGTTTTTTAGATAATTGGGTTTTTGCGTTTAGGAGGTTTT
 TATTTTTTGTTTGGGTAAAGGTAGTAGAGTTTCGGGAAAGGGATAGGGG
 GTTTAAGTGATGTTTTGGGGTATTGGCGTGGGAGAGTGGATTTTCGAAGT
 TGATAGATGGGTATTTTTTGACGGGGGGTAGGGGCGGAATTTGAGAGGC
 GTAAGGCGTTGTGAATTTTGGGGAGGGGGGTAGTTTGTAGGTCGCGAGG
 GAAGCGTTGAGGATTAGGAAGGGGGTATTGAGTGTTCTGGGGGAATTT
 TCGTGATAGGAATTGGAATATGTTTTGAGGGGGATATTATGTTTTTAAA
 ACGTCGTTGGTTATGAGGTTAGGAGTTTTAGATTAGTTTGATTAAACGTN
 GGTGAAATTTCTTTTTATTAATAAATAAATAAATAAATAAATAAATAA
 CGTTTTAGTTATTTAGGAGGTTGAGGTAGGAGAATCGTTAGAATTCGGGA
 GGCGGAGGTTGTAGTGAGTCGAGATCGCGTTATTGTATTTTAGTTTGGGC
 GATAGAGCGAGATTGTTTTAAAATAAAATAAAATAAAATAAAATAAAAA
 ATATCGGTTGGTATGTATGAGAGGATGGGATTTTGTGGAAGAAGAGGTG
 TTAGGAATATGTTTGGGAAGGGGAGGAGATAGGATTTTGTGGGAGGGAG
 AATTTAAGAATTGGATTTATTTGCGTTATTGAGAAAGCGTAAGAGGGAAG
 TAGAGGAGCGTTAGTAGTAATAGATGTTGTCTAGGGATGTGTTTGAGG

Figure A1.3: Methylated region of BRCA1 gene

AGGATTTAGAGATGAGAGTAGGTTATTGGGAAAGGTTAGGGGCGGGGGAG
 GTTTTGATTGGTGTGGTTTGGTCGTTGTTGATTTTGGTTTTATGTAAGGG
 AAAGAAAATAATTAGAAATATTGGAGAAAGTTAAGGTTATTATTATTTAT
 TCGGTTAGTTATTTTTTTGTAGTTTTTTTTTTTTTTGGAGAAAGGAAAAGAT
 TTAAGGGGTTGGTAGTAATATGTGAAAAAATTTAGAATTTATGTTGTTTA
 ATTATAAAAAGTAATTTTAGAATTTTAAAAATATAGGACGTTGTTATT
 AGTTTTTTGGTTTGTATTATTTTAAAATTTTTTAAATTTTAAATTTATTTTA
 TTTTAAAATGATAAAATGAAGTTGTTATTTTATAAATTTTTTAAAAAGAT
 ATATATATATGTTTTTTTAATGTGTTAAAGTTTATTGGAATAGAAAGAAA
 TGGATTTATTTGTTTTTCGCGTTGAAGAAGTATAAAATGTTATTAATGTTA
 TGTAGAAAATTTTAGAGTGTTTTATTTGGTAAGTTAGTATAAGAGTGTAT
 TAATTTGGGATTTTATGATTATTTTTTATGTAAATGAATAGAATTGATTT
 TATATATTAGGGAAGAAAAGATATGTTTAGTAAGATTAGGTTATTGTAAT
 TGTTGATTTTTTTAATTGAAGAATTTTAAAAATATAGAAAATGATTTTTTG
 TTTTTATTTATTTTGTTTTTTTTATTTTTTTTTTTTTTAAATATAAATTTTGT
 GGTTCGGGAAAGATAGGGATTTTGTTTTGATTGGTTTTGTATTGGGGTAG
 GAATTTAGTTTAGATTAATTGGTATTTTGGTTTTTTTTTAGTTTTAAAATA
 AGTTTTATTATTTGAAATGGTAAAATAAAATTATGGATGAGGTCGAGGGC
GGTGGTTATGTTTGTAATTTTAGTATTTTGGGAGGTAAAGGTGGTAGGA
 TTACGAGGTTAGGAGATCGAGATTATTTTGGTTAATATGGTGAAATTTTT
 TTTTATTAAAAATATAAAAATTAGTTGGGCGTAGTGGTATGTGTTTGTA
 ATTTAGTTATTTAGGAGGTTGAGGTAGGAGAATTATTTGAATTAGGAGG
 TAGATGTTGTTGTGAGTTAATATGGTATTATTGAATTTAGCGATAGAGTT
 AAATTTATTTTAAAAAAAAAAAAAAAAAAAAAAAAANAAATATGGATGATCG
 GTGTCGTTGAGAGGATAGGTATTTGGAAGAATTTTGTGTTGAAATTGGTT
 TTGTATATATAATGAAATTATATATTTATTTATATATAATGAAATGTAGA
 GGTTTTTTTTTTATATAGGATTTTGTCGAGAGGTTGGAGTGTAGTGGTGT
 TATTATAGTTTA

Figure A1.3: Continued

Unethylated region of BRCA1 gene is given in Figure A1.4.

TTGTTGGNTTGGGTGTTAGGNTTTTGATTGTTTGGGGTTGGGGGTGTGGG
 GTTTGTTGAGTTTGTGTTTATTTGGAATTTGTGTTGGTTGGTGAGTGTGT
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 GAGGGAGTTGGTTTTGGTTTTGGTTAGTTTAGAGAGGGGTTTTATAGTG
 TAGTGGTGGGTGAAGGGTTTTTTAGTATGGNTAGAATGGATGTAAAG
 NTGAGGAGGTGTTGAGAGTGAGTGAGGGTTGTTAGTATGTTGTTATTTTG
 TATTTTGAATTATAGATTTTTTAATTTTTTGGNGTTTTTTGTTTATTTGGTT
 TTTTAGAATATGAAGGGTTTTTTTATTTTGTATTAAAAATGATTAGTTGTT
 TGGAGATATGGAAAAAGTTGTTTTTTTTTTTTGTAGGATTTTTTTTTTGAA
 TTTTTTAAATTTTTTTAGTGTGATGTGATTTTATTTTGTAAATTTAGGT
 TGTTTTTTTATTAGTTTTTTGTTTTTTGGGGAGGTGGTAATGTAAAGATTG
 TTTGTTGTTAGTTTTGTGTTATTTTTGTGGGGTGAATTTAATATGGTGA
 TAAAGATAGTAATTAGTTTTGTTTTTTTGTTGTTTGTAAAGAAGATTGGT
 TTTTATTATTTGTTTTTTAAAAATGATTATTTTATTGATTGGTGGTGTGTTG
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 TTTTATAAGATTATTTGTTTTAGATTTTTGGGGTTGGATGGGAATTGTAGT
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 TATGAAAATATAATATTTTAGTTTATAATTGTTGATAAGTATAAGTGTGT
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 TATTGGATGTTTTTTTTTATAAGATTATAGTTTTTAAGGAATATTGTGGTG
 AAGATTTTTTATTTGTAAATGTATGTTGGAAATAATTATTTTTTTTTTATTT
 TTTAATAATTTTTATTATTTATTTATTGAAATTGGAGATTTTTATTAGG
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 GTGTTGAGGATTAGGAAGGGGGTATTGAGTGTTGTGGGGGAATTTTTGT
 GATAGGAATTGGAATATGTTTTGAGGGGGATATTATGTTTTTAAAAATGT
 TGGTTGGTTATGAGGTTAGGAGTTTTAGATTAGTTGATTAATGTNNGTG
 AAATTTTGTTTTTTATTAATAAATAAATAAATAAATAAATAAATAAATAAATT
 TAGTTATTTAGGAGGTTGAGGTAGGAGAATTGTTAGAATTTGGGAGGTGG
 AGGTTGTAGTGAGTTGAGATTGTGTTATTGTATTTTAGTTTGGGTGATAG
 AGTGAGATTGTTTTAAAATAAAATAAAATAAAATAAAATAAAATAAATAATT
 GGTGTTGTTATGTATGAGAGGATGGGATTTTGTGGAAGAAGAGGTGTTAGG
 AATATGTTTGGGAAGGGGAGGAGATAGGATTTTGTGGGAGGGAGAATTT
 AAGAATTGGATTTATTTGTGTTATTGAGAAAGTGTAAGAGGGGAAGTAGA
 GGAGTGTTAGTAGTAATAGATGTTGTGTTGGTAGGGATGTGTTTGAGGAGGA

Figure A1.4: Unmethylated region of BRCA1 gene

TTTAGAGATGAGAGTAGGTTATTGGGAAAGGTTAGGGGTGGGGAGGTTT
 TGATTGGTGTGGTTTGGTTGTTGTTGATTTTGGTTTTATGTAAGGGAAAG
 AAAATAATTAGAAATATTGGAGAAAGTTAAGGTTATTATTATTTATTTGG
 TTAGTTATTTTTTTGTAGTTTTTTTTTTTTTTGGAGAAAGGAAAAGATTTAA
 GGGGTTGGTAGTAATATGTGAAAAAATTTAGAATTTATGTTGTTTAATTA
 TAAAAAGTAATTTTTAGAAATTTTTAAAAATATAGGATGTTGTTATTAGTTT
 TTTGGTTTGTATTATTTTAAAATTTTTTAAATTTTTAAATTTATTTTATTTTA
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 TATATGTTTTTTTAATGTGTAAAGTTTATTGGAATAGAAAGAAATGGAT
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 AAAATTTTAGAGTGTTTTATTTGGTAAGTTAGTATAAGAGTGTATTAATTT
 GGGATTTTTATGATTATTTTTTATGTAAATGAATAGAATTGATTTTATATA
 TTAGGGAAGAAAAGATATGTTTAGTAAGATTAGGTTATTGTAATTGTTGA
 TTTTTTAAATTGAAGAATTTTAAAAATATAGAAAATGATTTTTTGTTTTTT
 ATTTATTTTGTTTTTTTTTATTTTTTTTTTTTTTAAATATAAATTTTGTGGTTG
 GGAAAGATAGGGATTTTGTTTTGATTGGTTTTGTATTGGGGTAGGAATTT
 AGTTTAGATTAATTGGTATTTTGGTTTTTTTTTTTAGTTTTAAATAAGTTTT
 ATTATTTGAAATGGTAAAATAAAATTATGGATGAGGTTGAGGGTGGTGGT
 TTATGTTTGTAATTTTAGTATTTTGGGAGGTTAAGGTGGTAGGATTATGA
 GGTTAGGAGATTGAGATTATTTTGGTTAATATGGTGAAATTTTTTTTTTAT
 TAAAAATATAAAAATTAGTTGGGTGTAGTGGTATGTGTTTGTAATTTTAG
 TTATTTAGGAGGTTGAGGTAGGAGAATTATTTGAATTAGGAGGTAGATGT
 TGTTGTGAGTTAATATGGTATTATTGAATTTTAGTGATAGAGTTAAATTTT
 ATTTTAAAAAAAAAAAAAAAAAAAAAAAAAANAATATGGATGATTGGTGTTGT
 TGAGAGGATAGGTATTTGGAAGAATTTTGTGTGAAATTGGTTTTGTATA
 TATAATGAAATTATATATTTATTTATATATAATGAAATGTAGAGGTTTTTT
 TTTTATATAGGATTTTTGTTGAGAGGTTGGAGTGTAGTGGTGTATTATAG
 TTTA

Figure A1.4: Continued

Appendix B: Homo sapiens RASSF1A (RASSF1A) gene, promoter region and partial cds.

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ATCCTAGAACCTTGGACCTCTAAGCCCTACTCCACATCCCCACATGCA
TTGCCATCCTCAATACCCACCTGCCTGCAGGGAGTGCCAAGTCAAGCACT
GGGAAAAGCATGGAAAGACTTGTGTCTTGGCAGCCCAGGGTGACAGAGC
CAAATGAGGGCTGCAGTTGCTGAGGGCCGACCACCCATGCCAAGGGAAT
CCACCCAGAATGCACCCCTGAACCTCAAGATCACCGGTCCAGCCTCTGCCG
GAGCCCCAGTCTCCGCAGTGGAGAGCAGAGCGGGCGGTAAAGCTGCTGA
CCGATCTCCCTCCTCCTCACCCCAAGTGAAGGCTCGAGACTTCCTGCCCC
ACCCAGTGGGTAGGCCAAGTGTGTTGCTTCAGCAAACCGGACCAGGAGG
GCCAGGGCCGGATGTGGGGACCCTCTTCCTCTAGCACAGTAAAGCTGGCC
TCCAGAAACACGGGTATCTCCGCGTGGTGCTTTGCGGTTCGCCGTCGTTGT
GGCCGTCCGGGGTGGGGTGTGAGGAGGGGACGAAGGAGGGAAGGAAGG
GCAAGGCCGGGGGGGGCTCTGCGAGAGCGCGCCAGCCCCGCCTTCGGGC
CCCACAGTCCCTGCACCCAGGTTTCCATTGCGCGGCTCTCCTCAGCTCCTT
CCCGCCGCCCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCGGGGGCCCGC
CCTGTGGCCCCGCCCCGGCCCGCGCTTGCTAGCGCCCAAAGCCAGCGAAG
CACGGGCCCAACCGGGGCCATGTGCGGGGGAGCCTGAGCTCATTGAGCTGC
GGGAGCTGGCACCCGCTGGGCGCGCTGGGAAGGGCCGCACCCGGCTGGA
GCGTGCCAAACGCGCTGCGCATCGCGCGGGGCACCGCGTGCAACCCACA
CGGCAGCTGGTCCCTGGCCGTGGCCACCGCTTCCAGCCCCGCGGGGGCCCGC
CACGCACACGCTGGTGCGACCTCTGTGGCGACTTCATCTGGGGCGTTCGTGC
GCAAAGGCCTGCAGTGCGCGCGTGAGTAGTGGCCCCGCGCGCCTACGAG
AGCGGAAGGGGCAGCCAAGGGGCAGCGCAGTCCGCCGCGGGTCAAGTCG
CGGCAGAGGGGGTTCGGCGGGGACAGCTCCCGAGGACTAGGTCCGTTACT
TTCGCCCCATCGCTGAAGAGTGCGCGAAAATGGTTTATCCCTTGTCGCAC
TCCACTCGTATCTGGGCCACAGATGAGCAGAGGTGGCTGCTTATATGTAA
AAATACGCTGATTTTAAGTTTCTTATCTTTAAAATGCCTTGGCCCTTCTTG
AGAAAGGGTTTGTGCCTACTGTCCTCGGAGTCCATCTTCCCAGGCTTGCC
TCTTCTCAAACACTCATGACCCCTCCAGAACCTTTAGGGTGAAGGGAAA
TTACCACCTATGGGAGGGAGCCTGGAAAAATTTAGAACCTTTGGTGGGGC
CCCTGCAAGCAGGAGTTTGTGTGAGTCTTTATTTAGCAAACACCCTTTTCT
GACCCAGTGAATCAGATGCTAAAATATGCACGCAGCCACACACCCAGCA
GTCCTTCTGCACCCCTGGGAATCGCCAGCAAGCAAAGGTTGCTCTCCCCT
GGGTAGACACCAGCTGGAATCACCAGGGGTGCTTTTACAGTCCTCCCCGC
TAGCCTGGATCCCACCGCAGACCTGTTGAATCAACTGCTGGGAGTGGACC
CTAGGCATCAGTAAATTTTAAAAACTCCCCAAATTATTGTAACATGGAGT
CTGGGTTGAGCATCACTGCTCTGGCCTATTTAGGAACTTGTGGATGGATA
GTGTCCCAGGTCTGTGTGTGCATGGAGACCCTCTCATCCGGTACAAGAGG
ACATCACAAATTCAGCTGGGGGGAGCACAAAGTTGTGACAGAATGCAAA
GAATGAACAAGGGGCCGAGCGCGGTGGCTCATGCCTGTAATCCCAGCAC
TTCGGAAGGCCGAGGCCGGGTGGATCACCTGAGGTCAGGAGTTCAAGACC
AGCCTGGCCAACATGGTG

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Figure B1.1: Homo sapiens RASSF1A (RASSF1A) gene, promoter region and partial cds. (ACCESSION: DQ444319)

GC% of RASSF1A promoter region is given in Figure B1.2. It was determined by website (<http://canna.cau.ac.kr/util/cgi-web/index.html>).

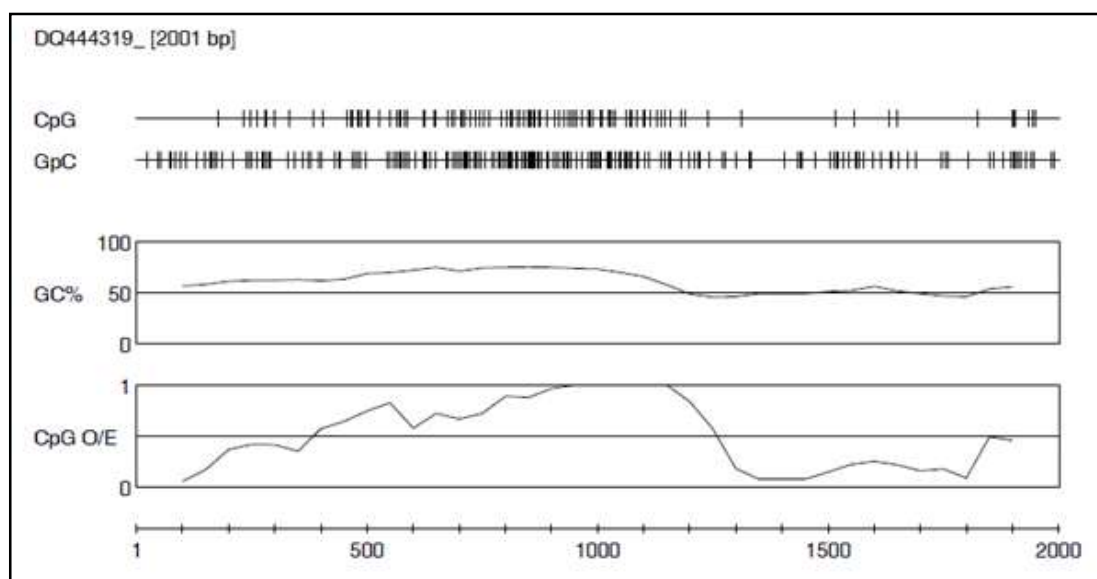


Figure B1.2: GC% of RASSF1A promoter region

Primers details are given in below.

RASSF1A-F GGGACTTTGAGTTTATTGAGTTG

RASSF1A-R CCACAACCAAAAACCAACTACC (Reverse Complementer-
GGTAGTTGGTTTTTGGTTGTGG)

Methylated region of RASSF1A gene is given in Figure B1.3.

AGTATAGTAAAGTTGGTTTTTAGAAATACGGGTATTTTCGCGTGGTGT
 GCGGTCGTCGTCGTTGTGGTCGTTCCGGGGTGGGGTGTGAGGAGGGGACG
 AAGGAGGGAAGGAAGGGTAAGGCCGGGGGGGGTTTTGCAGAGAGCGCGTT
 TAGTTTCGTTTTTCGGGTTTTATAGTTTTGTATTTAGGTTTTATTGCGCGG
 TTTTTTTAGTTTTTTTTTCGTCGTTTAGTTTGGATTTTGGGGGAGGCCGTTGA
 AGTCGGGGTTCGTTTTGTGGTTTCGTTCCGGTTCGCGTTTGTAGCGTTTAA
 AGTTAGCGAAGTACGGGTTTAATCCGGGTTATGTCGGGGAGTTTGAGTTT
 ATTGAGTTGCGGGAGTTGGTATTCGTTGGGCGCGTTGGGAAGGGTTCGTAT
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 ATTTTATACGGTAGTTGGTTTTTGGTTCGTGGTTATCGTTTTTAGTTCGCGG
 GGTTTCGTTACGTATACGTGGTGCGATTTTTGTGGCGATTTTATTTGGGGCG
 TCGTGCGTAAAGGTTTGTAGTGCGCGCGTGAGTAGTGGTTTCGCGCGTTT
 ACGAGAGCGGAAGGGGTAGTTAAGGGGTAGCGTAGTCGTCGCGGGTTAA
 GTCGCGGTAGAGGGGGTCCGGCGGGGATAGTTTTTCGAGGATTAGGTTTCGTT
 ATTTTCGTTTTATCGTTGAAGAGTGCGCGAAAATGGTTTATTTTTTGTCTG
 ATTTTATTCGTATTTGGGTTATAGATGAGTAGAGGTGGTTGTTTATA

Figure B1.3: Methylated region of RASSF1A gene

Unmethylated region of RASSF1A gene is given in Figure B1.4.

AGTATAGTAAAGTTGGTTTTTAGAAATATGGGTATTTTGTGTGGTGT
 GTGGTTGTTGTTGTTGTGGTTGTTTGGGGTGGGGTGTGAGGAGGGGATGA
 AGGAGGGAAGGAAGGGTAAGGTGGGGGGGGTTTTGTGAGAGTGTGTTTA
 GTTTTGTTTTTGGGTTTTATAGTTTTGTATTTAGGTTTTATTGTGTGGTT
 TTTTTAGTTTTTTTTTGTGTTTAGTTTGGATTTTGGGGGAGGTGTTGAAG
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 TAGTGAAGTATGGGTTTAATTGGGTTATGTTGGGGAGTTTGAGTTTATT
 GAGTTGTGGGAGTTGGTATTTGTTGGGTGTGTTGGGAAGGGTGTATTTG
 GTTGGAGTGTGTTAATGTGTTGTGATTGTGTGGGGTATTGTGTGTAATTT
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 GTTATGTATATGTGGTGTGATTTTTGTGGTGATTTTATTTGGGGTGTGTG
 TGTAAGGTTTGTAGTGTGTGTGTGAGTAGTGGTTTTGTGTGTTTATGAG
 AGTGGAAGGGGTAGTTAAGGGGTAGTGTAGTTGTTGTGGGTAAAGTTGTG
 GTAGAGGGGGTGGTGGGGATAGTTTTTGAGGATTAGGTTTGTATTTTT
 GTTTTATTGTTGAAGAGTGTGTGAAAATGGTTTATTTTTTGTGTATTTA
 TTTGTATTTGGGTTATAGATGAGTAGAGGTGGTTGTTTATA

Figure B1.4: Unmethylated region of RASSF1A gene

Appendix C: Homo sapiens retinoic acid receptor beta (RAR β)

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TATTTTGTTTTTTTAGTTAATATTGACGTTGGGTAGTATTATATGTTTATTT
TATAGATGAGGAAATTGAAGTTTGAAGAAGTTAAATATTTATTTTAGAAT
ATATAGTTGGTAGTGGTAGATTGGAATTGGAATTTAGTTTAGTTTGATT
TTAATTTATGTTTTTGATTATTATATTGTTTTTTAAGTTTAGATTGAAA
TTTTATTTTTTGTGTGGTTGTGTGTTTGGGATAGGGGTAAATTTTTGA
TTATTTTATATGTTGTATAGAATTTGGAGAGGATTTTTTAAAGTAAATGA
ATTTTCGAAAGTTGGATTGTAGAGTAAACGAGTGTAGTTAATTTAGTTAGG
GGTTTGTAAAGAGGGAGAAAGAGAAAAAGATTGTGGAATGGAAAGTTTTT
TAATTTAAGTTTTTTTTTAAGGGGTAGTTATTTTTTGTTTTATAGTTTAGGGT
TTGTATGTGTTTTTTTTTGGAGTGGAAAAATATATAAGTTATAAGGAATTT
AATAGATAGAAAGGCGTATAGAGGAATTTAAAGTGTGGGTGGGGGGCG
AGGCGGTGGGCGGGAGGCGAGCGGGCGTAGGCGGAATATCGTTTTTTAA
GTTAAGTCGTCGTAAATAAAAAGGCGTAAAGGGAGAGAAGTTGGTGT
AACGTGAGTTAGGAGTAGCGTTTCGGTTTTTTTTTTGTTTATTTTAAAGT
ATTTTTTGTATTGTTTTTAAGGTGAGAAATAGGAAAGAAAACGTGGTTT
GTGCGTTTCGTTGTTTGTTTTTTGGTTGTTTGTGTTTGTAGGGTTGTTGGGA
GTTTTTAAGTTTTGTGAGAATTTTGGGAGTTGGTGATGTTAGATTAGTTGG
GTTATTTGAAGTTAGTAGTTTCGGGTAGGGTTTATCGAAAGTTTATTCGT
ATATATTAGGTAATTTAATTTTTTATTTTGTGTGATAGAAGTAGTAGGAA
GTGAGTTGTTTAGAGGTAGGAGGGTTTATTTTTTGTAAAGGGGGGATTA
GAATTTTTTTATGCGAGTTGTTTGAAGATTGGGATGTGAGAACGCGAGC
GATTCGAGTAGGGTTTGTGTTGGGTATCGTCGGGGTAGGATTCGGAACGTA
TTCGGAAGGTTTTTTGTAAGTATTTATTTGGAAGGAGAATTTGGGATTTT
TTGGGAATTTTTTCGTTTCGGTTGGATTGGTCGAGTAAGTTTGGAATATGG
TAAATGATTATTTGGATTAAATATAGGTTTTTAGTTGGTTTGTGTTTATA
ATTTATGATTTCGGGGTTGGGAAAAAGATTAATAGTTTACGTGTTAAAAAA
GGGGTAGAGTTTGATGGAGTTGGGTGGATTTTTTTATGTTATTTGTTTTA
TATTTAGAGGATAAGTATTTTTGTAGATATTTAGTGTAAGGGAGATTATG
TTTGATTGTATGGATGTTTTGTTAGTGAGTTTGGGTAAATTTTGGATTTT
TATATTGCGAGTTCGTTTTTTGTATGTTTATAGGAGA
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Figure C1.1: Homo sapiens retinoic acid receptor beta (RAR β) (LOCUS NG_029013 775610 bp DNA linear PRI 12-SEP-2016, RefSeqGene on chromosome 3)

GC% of RAR β promoter region is given in Figure C1.2. It was determined by website (<http://canna.cau.ac.kr/util/cgi-web/index.html>).

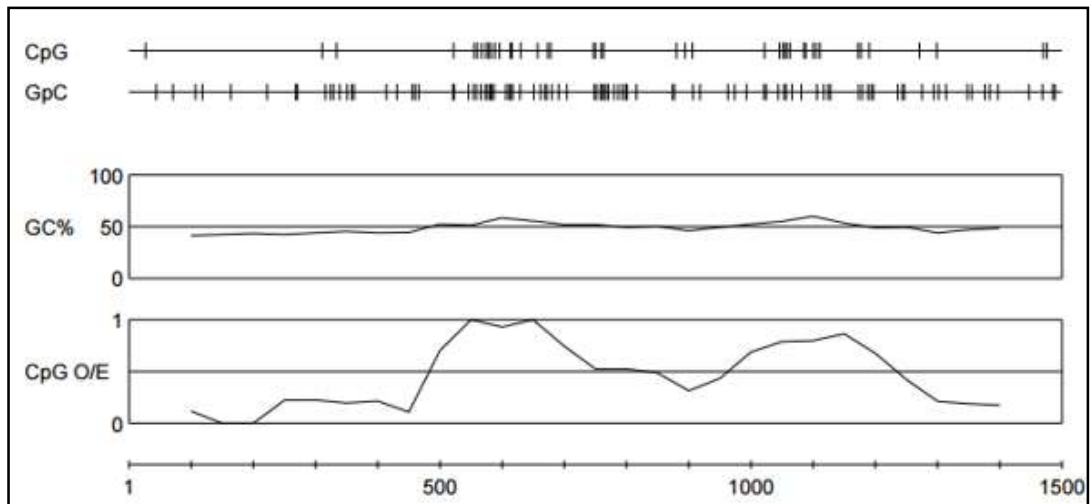


Figure C1.2: GC% of RAR β promoter region

Primers details are given in below.

RAR β -F AGTTGTTTGAGGATTGGGAT

RAR β -R TCTCCTCCAAATAAATACTTACA (Reverse Complement-
TGTAAGTATTTATTTGGAAGGAGA)

Methylated region of RAR β gene is given in Figure C1.3.

```

ATAAGTTATAAGGAATTTAATAGATAGAAAGGCGTATAGAGGAATTTAA
AGTGTGGGTTGGGGGGCGAGGCGGTGGGCGGGAGGCGAGCGGGCGTAG
GCGGAATATCGTTTTTTTAAGTTAAGTCGTCGTAAATAAAAAGGCGTAAAG
GGAGAGAAGTTGGTGTTTAACGTGAGTTAGGAGTAGCGTTTCGGTTTTTT
TTTTGTTTATTTTAAAAGTATTTTTTGTATTGTTTTTAAAGGTGAGAAATAG
GAAAGAAAACGTCGGTTTTGTGCGTTTCGTTGTTTGTTTTTTTGGTTGTTTGT
TTTTGTAGGGTTGTTGGGAGTTTTTAAGTTTTGTGAGAATTTTGGGAGTTG
GTGATGTTAGATTAGTTGGGTATTTTGAAGGTTAGTAGTTCGGGTAGGGT
TTATCGAAAGTTTATTCGTATATATTAGGTAATTTAATTTTTTATTTTGTGT
GATAGAAGTAGTAGGAAGTGAGTTGTTTAGAGGTAGGAGGGTTTATTTTTT
TGTTAAAGGGGGGATTAGAATTTTTTTATGCGAGTTGTTTGAGGATTGGG
ATGTCGAGAACGCGAGCGATTTCGAGTAGGGTTTGTTTGGGTATCGTCGGG
GTAGGATTCGGAACGTATTCGGAAGGTTTTTTTGTAAGTATTTATTTGGAA
GGAGAATTTGGGATTTTTTTGGGAATTTTTTCGTTTCGGTTGGATTGGTCTGA
GTAAGTTTGGAAAATGGTAAATGATTATTTGGATTAA
  
```

Figure C1.3: Methylated region of RAR β gene

Unmethylated region of RASSF1A gene is given in Figure C1.4

```
ATAAGTTATAAGGAATTTAATAGATAGAAAGGTGTATAGAGGAATTTAA
AGTGTGGGTTGGGGGGTGAGGTGGTGGGTGGGAGGTGAGTGGGTGTAGG
TGGAATATTGTTTTTTAAGTTAAGTTGTTGTAAATAAAAAGGTGTAAAGG
GAGAGAAGTTGGTGTTTAATTGTGAGTTAGGAGTAGTGTTTTGGTTTTTTT
TTGTTTTATTTTAAAAGTATTTTTTGTATTGTTTTTAAGGTGAGAAATAGGA
AAGAAAATGTTGGTTTGTGTGTTGTTGTTTGTTTTTTTGGTTGTTTGT
TGTAGGGTTGTTGGGAGTTTTTAAGTTTTGTGAGAATTTTGGGAGTTGGT
GATGTTAGATTAGTTGGGTTATTTGAAGGTTAGTAGTTTGGGTAGGGTTT
ATTGAAAGTTTATTTGTATATATTAGGTAATTTAATTTTTTATTTTGTGTG
ATAGAAGTAGTAGGAAGTGAGTTGTTTAGAGGTAGGAGGGTTTATTTTTT
GTTAAAGGGGGGATTAGAATTTTTTTATGTGAGTTGTTTGAGGATTGGGA
TGTTGAGAATGTGAGTGATTGAGTAGGGTTTGTTTGGGTATTGTTGGGG
TAGGATTTGGAATGTATTTGGAAGGTTTTTTGTAAGTATTTATTTGGAAG
GAGAATTTGGGATTTTTTTGGGAATTTTTTGTGTTTGGTTGGATTGGTTGAG
TAAGTTTGGAAAATGGTAAATGATTATTTGGATTAA
```

Figure C1.4: Unmethylated region of RAR β gene