SINGLE NUCLEOTIDE POLYMORPHISM IN THE SEED REGION OF MICRORNA ALTERS THE EXPRESSION OF ITS MATURE MICRORNA

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ABSTRACT

MicroRNAs (miRNA) are a class of non-coding RNAs with approximately 18 to 28 nt, which are important in post-transcriptional gene regulation. In this study, potential single nucleotide variant within seed region sequence of mature miRNAs of *Homo sapiens* was identified. Mature miRNA sequences of *Homo sapiens* were collected from miRBase database. A total of 1344 mature miRNA sequences were used to compare with single nucleotide variant sequence in UCSC database to search for candidate miRNA variant. Fourty-two mature miRNAs that have single nucleotide variant on seed-region were identified. Prediction of the secondary structure with MFOLD had identified a total of fourteen potential miRNAs that have a single nucleotide variant on the seed region. Two mature miRNAs with SNP on their seed-region had been chosen to validate experimentally with real-time PCR approach; miR124-3 (miRBase ID MI0000445) and miR-662 (miRBase ID MI0003670). These two potential single nucleotide variant on the seed region sequence of mature miRNAs were found to enhance the expression of its mature miRNA. The nucleotide change on the seed region of miR124-3 and miR-662 is suspected to provide stable secondary structure of pri-miRNA and pre-miRNA compare to the wild-type allele of miRNAs. The increased expression of specific miRNAs could possibly render the miRNA to act as an oncomiR or vice versa as a tumour suppressor miRNA, hence this study emphasizes that a single nucleotide change on the seed-region of mature miRNA could potentially lead to widespread phenotypic effects and broadens our understanding on miRNA biogenesis.

ABSTRAK

MicroRNAs (miRNA) adalah kelas bukan pengekodan pernas dengan kira-kira 18-28 nt, yang penting dalam peraturan gen pasca-transkripsi. Dalam kajian ini, potensi varian tunggal nukleotida dalam urutan rantau benih miRNAs matang Homo sapiens telah dikenal pasti. MiRNA urutan matang Homo sapiens telah dikumpulkan dari pangkalan data miRBase. Sebanyak 1344 urutan miRNA matang telah digunakan untuk membandingkan dengan urutan varian nukleotida tunggal dalam pangkalan data UCSC untuk mencari calon miRNA varian. Empat puluh dua miRNAs matang yang mempunyai varian nukleotida tunggal pada benih rantau telah dikenalpasti. Ramalan struktur menengah dengan MFOLD telah mengenal pasti sebanyak empat belas miRNAs potensi yang mempunyai varian tunggal nukleotida pada rantau benih. Dua miRNAs matang dengan SNP pada benih-kawasan telah dipilih untuk mengesahkan uji kaji dengan pendekatan PCR masa sebenar; miR124-3 (miRBase ID MI0000445) dan Mir-662 (miRBase ID MI0003670). Kedua-dua varian potensi tunggal nukleotida pada urutan rantau benih miRNAs matang telah didapati untuk meningkatkan ungkapan miRNA matang itu. Perubahan nukleotida kepada rantau benih daripada miR124-3 dan mir-662 disyaki menyediakan struktur menengah stabil pri-miRNA dan pra-miRNA berbanding allele liar-jenis miRNAs. Ungkapan peningkatan miRNAs tertentu mungkin boleh menyebabkan miRNA untuk bertindak sebagai oncomiR atau sebaliknya sebagai penindas miRNA tumor, maka kajian ini menekankan bahawa perubahan nukleotida tunggal pada benih rantau miRNA matang berpotensi membawa kepada kesan fenotip meluas dan meluaskan pemahaman kita pada biogenesis miRNA.

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ABBREVIATIONS

%	precent sign
~	approximate
Δ	delta
°C	degree Cesiues
BLAST	basic local alignment seach tool
BLAT	BLAST-like alignment tool
bp	base pair
CD164	Sialomucin core protein 24
CDK6	Cyclin-dependent kinase 6
CREB5	Cyclic AMP-responsive element-binding protein 5
CSF3	colony stimulating factor 3
DCC	Deleted in Colorectal Cancer
DEPC	Diethyl pyrocarbonate
DNA	deoxy ribonucleic acid
dNTP	deoxyribonucletide triphosphate
dsRNA	double-stranded ribonucleic acid
e.g.	*exempli gratia*
et al	*et alia*
etc	*et cetera*
g	gram
HEK293T	human embryonic kidney 293T
IGFBP7	Insulin-like growth factor-binding protein 7
ITGB1	Integrin Beta 1
kcal/mol	kilocalorie/mole
M	molar
MFE	minimal folding energy
MFEI	minimal folding free energy index
min	minute
miRNA	microRNA
MKX	Homeobox protein Mohawk
ml	milliliter
mm	millimetre
mM	millimolar

mRNA	messenger ribonucleic acid
NEGR1	Neuronal growth regulator 1
nm	nanometer
nt	nucleotide
OSCC	oral squamous cell carcinoma
PCR	polymerase chain reaction
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTPN12	Tyrosine-protein phosphatase non-receptor type 12
qRTPCR	quantitative real time PCR
RNA	ribonucleic acid
RQ	relative quantification
SNP	single nucleotide polymorphism
ssRNA	single-stranded ribonucleic acid
UTR	untranslated region
UV	ultraviolet
VAMP3	Vesicle-associated membrane protein 3
µl	micro liter

CHAPTER 1 INTRODUCTION

1.1 Introduction

In 1990s, two research groups - Lee *et al.* (1993) and Wightman *et al.* (1991) have independently discovered a small 22 nt RNA from the lin-4 gene. They found that lin-4 gene sequence complement that of the lin-14 gene, and negatively regulates lin-14 expression in *Caenorhabditis elegans*. These classes of small non-coding RNAs are later known as microRNAs (miRNAs). In general, miRNAs function to downregulate gene expression by cleaving or inhibiting the translation of their target gene transcript. They are important in signaling, cell fate identity, organ differentiation and development, stress responses, disease and carcinogenesis (Lai, 2003). Up to date, more than 24,521 miRNAs have been identified from 133 species including primates, rodent, birds, fish, worms, flies, plant and viruses (miRBase Release 20.0, June 2013).

Hypothetically, defect in miRNA biogenesis or aberrant miRNA expression level may lead to disorganized cellular processes and eventually cause human diseases. For example, miR-107 was significantly down regulated in Alzheimer's disease (Garofalo *et al*., 2008; Nelson *et al*., 2008); miR-133b show very low expression level in Parkinson's disease and miR-1 is over expressed in individuals with coronary artery disease (Garcia and Miska, 2005; Rooij *et al*., 2006).

Single nucleotide polymorphisms (SNPs) are the most common DNA variation found in human genome and contribute to human disorders or cancers. Single nucleotide variation within the seed region of a mature miRNA sequence may contribute to a different expression of miRNA, including post transcription miRNA processing and miRNA stability and functions. Besides that, nucleotides variation

within the seed region of mature miRNA that are phenotypic neutral may contribute to the evolution of miRNAs with new or altered function that resulted in the production of novel miRNAs with more specialized functions (Sun *et al*., 2010).

This study on single nucleotide variant within the seed region of mature miRNA sequence ultimately explored the post-processing of the affected miRNA. The knowledge gained from this study provides insights into how SNPs within seed regions may influence the biogenesis of a miRNA.

1.2 Literature Review

1.2.1 Organization of human genome

A genome is an organism's complete set of double stranded deoxyribonucleic acid (DNA). Each genome contains important information to build and to maintain one organism. In human, the entire genome consists of approximately 3.2 billion DNA base pairs which are organized in 24 distinct chromosomes (Vogel and Motulsky, 1997; Venter *et al*., 2001; Bonham *et al*., 2005). Human genome project has revealed that there are about 30,000 genes (two percentage of the total DNA content) that are responsible for coding ribonucleic acid (RNA). RNA involves in producing functional proteins, such as coding, structural and regulatory (Vogel and Motulsky, 1997; Venter *et al*., 2001; Collins, 2003; Kidd *et al*., 2004; Bonham *et al*., 2005; Sampo Sammalisto, 2008).

One individual receives one chromosome of each type from each parent. Therefore, each individual has its own sets of 22 autosomal chromosome pairs (Figure 1-1) and a pair of sex chromosome, either XX (female) or XY (male) (Vogel and Motulsky, 1997; Sampo Sammalisto, 2008). Majority of the human genetic information

is identical among all human beings, yet each individual's genetic code still differs slightly. It has been shown that any two human individuals that are about 99.8% to 99.5% genetically identical, they share only the most important genetic material. The remaining 0.2% to 0.5% difference, which represents sixteen to fifteen million gene variant, can occur in different combinations which are enough to cause individual uniqueness (Venter *et al*., 2001; Collins, 2003; Kidd *et al*., 2004; Bonham *et al*., 2005).

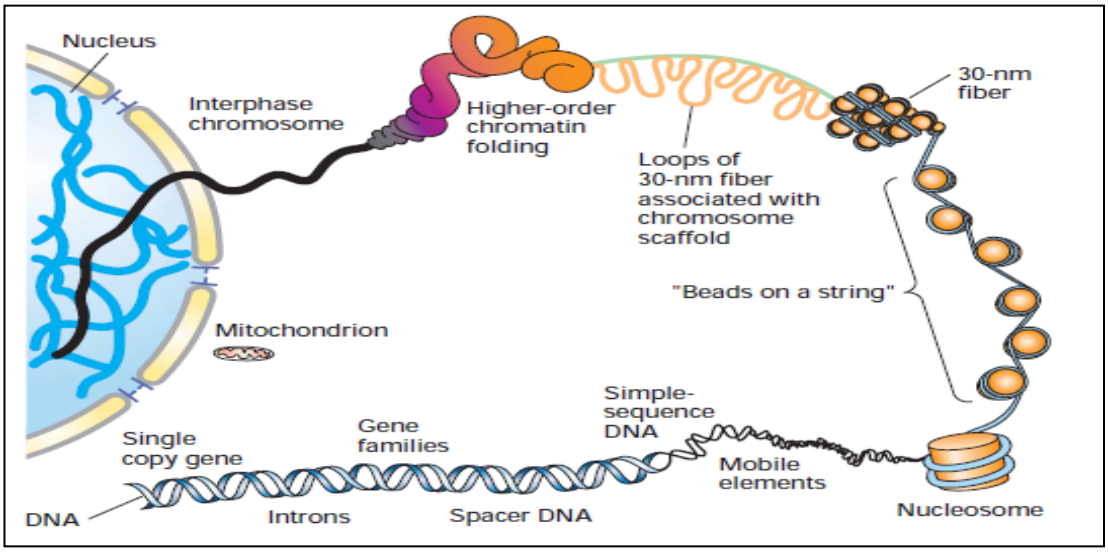


Figure 1-1: Structure of DNA and chromosome. Double stranded DNA is tightly coiled within each chromosome.
(Figure from Sharma, N.S., 2005 - Molecular Structure of Genes and Chromosomes)

1.2.1.1 Human genetic variation

Genetic variation slowly leads to the genetic diversity of human beings (Vogel and Motulsky, 1997; Brooker, 2005; Li, 2012). It represents the total amount of genetic characteristic obtained within human. The genetic differences that occurred in between human can be found in both individual and population level (Snustad and Simmons, 2003; Jorde *et al*., 2004; Kidd *et al*., 2004; Brooker, 2005; Frazer *et al*., 2009). In the individual level, there is no two humans with identical genetic. Even monozygotic twins have genetic differences due to copy number variation and mutations. In population level, alleles occur at different frequencies in different population that is especially

geographically and ancestrally divert (Vogel and Motulsky, 1997; Snustad and Simmons, 2003; Jorde *et al*., 2004; Wong *et al*., 2007; Frazer *et al*., 2009).

Genetic variation in human genome exists as various levels, from single base pair to thousand kilo base pairs (Snustad and Simmons, 2003; Brooker, 2005; Wong *et al*., 2007). The causes of these variations are the genetic recombination, random assortment, mutational event and evolutionary forces (natural selection and genetic drift) (Vogel and Motulsky, 1997; Snustad and Simmons, 2003; Svetlanov and Cohen, 2004; Brooker, 2005; Sampo Sammalisto, 2008; Frazer *et al*., 2009). There are common and rare variant: common variant are synonymous with polymorphisms with a minor allele frequency of at least one percent, while rare variant are those with a minor allele frequency less than one percent (Vogel and Motulsky, 1997; Snustad and Simmons, 2003; Frazer *et al*., 2009). In addition, genetic variant have been discussed in the terms of nucleotide composition – single nucleotide variant and structural variant (Figure 1-2). The vast majority of genetic variant are hypothesized to be neutral that do not contribute to phenotypic variation (Vogel and Motulsky, 1997; Snustad and Simmons, 2003; Brooker, 2005; Frazer *et al*., 2009).

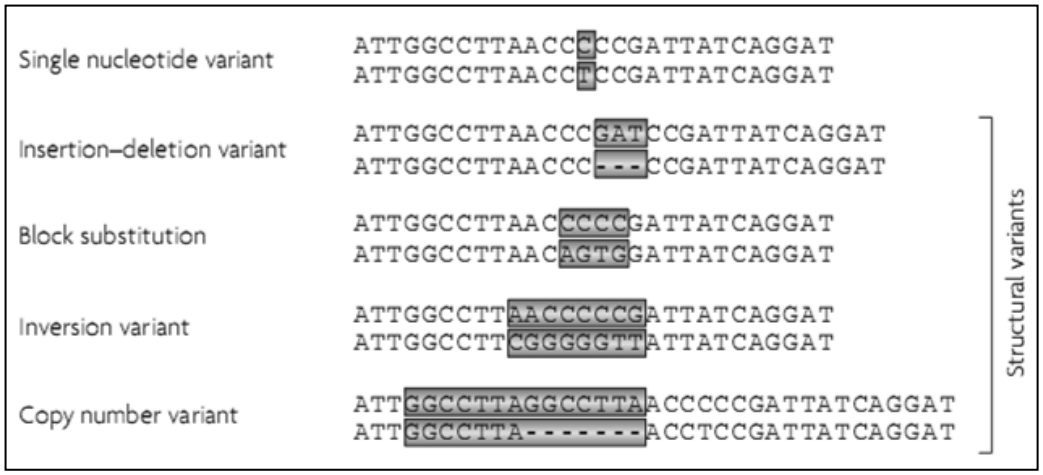


Figure 1-2: Classes of human genetic variant. (Figure from Frazer et al., 2009)

1.2.1.2 Single nucleotide variant

Single nucleotide variant are classified when a single nucleotide (adenine-A, thymine-T, cytosine-C or guanine-G) is altered in the DNA sequence (Vogel and Motulsky, 1997; Snustad and Simmons, 2003; Brooker, 2005; Frazer *et al*., 2009). Single nucleotide polymorphisms (SNPs) are the most common single nucleotide variation among individuals (Figure 1-3). SNPs are estimated to occur at 1 out of every 1,250 bases in human genome (Vogel and Motulsky, 1997; Syvänen, 2001; Venter *et al*., 2001; Sampo Sammalisto, 2008; Frazer *et al*., 2009). Survey of the sequencing results show that there are at least 30 million SNPs occur in human genome (Venter *et al*., 2001; Duan *et al*., 2007; Frazer *et al*., 2009). There are almost two million of validated SNPs in public available databases – SNP Consortium, International Hapmap Project, UCSC Genome Bioinformatics site and National Center for Biotechnology Information (NCBI) (Syvänen, 2001; Kidd *et al*., 2004; Frazer *et al*., 2009).

SNPs are innumerable rare and novel, in some cases segregate only in a nuclear family or a single individual (Frazer *et al*., 2009). Only a small proportion of SNPs (<1%) give impact at the phenotypic level (Syvänen, 2001; Venter *et al*., 2001). This is because only SNPs that occur in the coding regions and regulatory regions of the genes will alter the amino acid sequences of proteins and gene expression. These alterations cause most of the dominantly or recessively inherited monogenic disorders (Vogel and Motulsky, 1997; Syvänen, 2001; Venter *et al*., 2001; Snustad and Simmons, 2003; Brooker, 2005; Li, 2012). Therefore, these SNPs are routinely analyzed for diagnostic and pharmacogenetic purposes. However, SNPs that located in the non-coding regions of the genome are yet to study their impact on phenotype (Vogel and Motulsky, 1997; Syvänen, 2001; Brooker, 2005; Li, 2012). SNPs are useful as markers in common and

multifactorial disorders, besides population genetics and evolutionary studies (Syvänen, 2001; Kwok and Chen, 2003; Snustad and Simmons, 2003; Brooker, 2005).

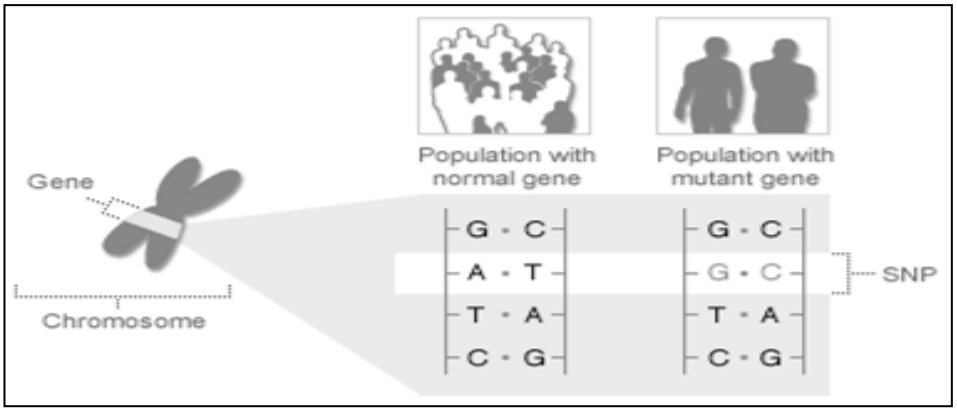


Figure 1-3: Single nucleotide polymorphisms (SNPs) in human population. (Figure from http://www.celera.com/celera/cdx_discover)

1.2.2 MicroRNA (miRNA) in human

To date, miRNAs have been found in plant and animal multicellular organisms (Carrington and Ambros, 2003; Millar and Waterhouse, 2005). According to the miRNA Registry Database – Release 20.0, June 2013 (http://www.sanger.ac.uk), 24,521 miRNA genes have been identified in 133 species, such as plant, primates, metazoan, vertebrates, amphibian, arthropoda, mammalian, DNA viruses, and single cellular algae. These include 1872 *Homo sapiens* miRNAs, 223 *Caenorhabditis elegans* miRNAs, 238 *Drosophila melanogaster* miRNAs, 346 *Danio rerio* miRNAs, 734 *Gallus gallus* (chicken) miRNAs and etc (Jones, 2004; Jones *et al*., 2006; Jones *et al*., 2008). Animal miRNAs are evolutionarily conserved for at least 400 million years old (Millar and Waterhouse, 2005).

MicroRNAs are produced to provide essential regulation of developmental processes (Bartel, 2004; Millar and Waterhouse, 2005; Kusenda *et al*., 2006; Meola *et al*., 2009; Kunej *et al.*, 2012). These miRNAs have the length of about 18-28 nt which

are excised from endogenously encoded hairpin RNAs. MicroRNA negatively regulate their 3' UTR target genes by translational inhibition or degradation of mRNA (Ambros, 2004; Cullen, 2004; Lippman and Martienssen, 2004; Gregory and Shiekhattar, 2005; Millar and Waterhouse, 2005; Lee *et al*., 2006; Saetrom *et al*., 2006; Zambon *et al*., 2006; Reddy *et al*., 2007; Asikainen *et al*., 2008; Kunej *et al.*, 2012). Significant numbers of human miRNAs are encoded within the introns of precursor mRNAs (Ambros, 2004; Bartel, 2004; Lee *et al*., 2004; Du and Zamore, 2005; Kusenda *et al*., 2006; Zeng, 2006; Kim *et al*., 2007; Meola *et al*., 2009). These intron-coded miRNAs are in the same orientation as the pre- mRNA, which suggest each processed transcript will produce one translatable mRNA and one functional miRNA (Millar and Waterhouse, 2005; Kim and Nam, 2006).

1.2.2.1 Characteristic of microRNA

There are several structural characteristics that are conserved among all miRNAs to distinguish them from random or genomic hairpins of similar lengths (Ambros *et al*., 2003; Saetrom *et al*., 2006; Kim and Nam, 2006; Zhang *et al*., 2006). It must follow certain features in order to be classified as miRNA. First, the 18-28 nt of mature miRNA sequence should be found within one arm of the hairpin structure that is lack of large bulges or internal loops. The stem-looped secondary structures of miRNAs are well conserved in humans (Ambros *et al*., 2003; Kim and Nam, 2006; Zhang *et al*., 2006; Kunej *et al.*, 2012). Secondly, the stable precursor hairpins have multiple foldings with the lowest negative minimal folding free energy (MFE) and highest negative minimal folding free energy index (MFEI) compared to other RNAs (Ambros *et al*., 2003; Bonnet *et al*., 2004; Zhang *et al*., 2006; Zhang *et al*., 2007; Kunej *et al.*, 2012). Several parameters were considered to obtain MFEI: adjusted minimal folding free energy (combination of minimal folding free energy and length of pre-miRNA), length

of RNAs and GC content. Most pre-miRNAs had an MFEI greater than 0.95 kcal/mol while other RNAs ranged between 0.5-0.7 kcal/mol (Ambros *et al*., 2003; Bonnet *et al*., 2004; Layton *et al*., 2005; Zhang *et al*., 2006). Third, these small miRNAs are believed to be evolutionarily or phylogenetically conserved in closely related species (Ambros *et al*., 2003; Kim and Nam, 2006; Saetrom *et al*., 2006; Zeng, 2006**)**. Fourth, all miRNAs have high complementarity to their targeted 3' UTR mRNA, either precise complementarity in plant or imperfect complementarity in animals. In animal miRNAs, Watson–Crick base pairing between six or seven nucleotides in the target mRNA's 3′UTR and nucleotides 2–7 or 2–8 (the "seed sequence") of the miRNA's 5′ end is required (Du and Zamore, 2005; Kim and Nam, 2006; Zhang *et al*., 2006). As a result, a single miRNA can regulate hundreds or thousands of different 3' UTR mRNA targets. Conversely, several miRNAs can bind cooperatively to a single 3' UTR mRNA target (Du and Zamore, 2005; Kim and Nam, 2006; Lee *et al*., 2006; Zhang *et al*., 2006).

1.2.2.2 MicroRNA biogenesis

MicroRNA resides in the intergenic (40%) or intragenic (60%) regions. It is an endogenous transcript that can self-complement to form imperfect stem loop structures (Ambros, 2004; Bartel, 2004; Cullen, 2004; Gregory and Shiekhattar, 2005; Lee *et al*., 2006; Saetrom *et al*., 2006; Zambon *et al*., 2006; Asikainen *et al*., 2008; Meola *et al*., 2009). A single miRNA is located on one arm of a hairpin precursor transcript. Sometimes one pri-miRNA transcript consists of several hairpins that give rise to different miRNAs which are known as polycistronic miRNA transcripts (Ambros *et al*., 2003; Du and Zamore, 2005; Millar and Waterhouse, 2005; Kim and Nam, 2006; Kim and Kim, 2007; Meola *et al*., 2009).

Initially, miRNAs are transcribed from long primary transcripts (pri-miRNAs) that varies from 400-3,000 bases by RNA polymerase II enzyme. Primary transcript is 5' capped and polyadenylated (Bartel, 2004; Du and Zamore, 2005; Kim and Nam, 2006; Kusenda et al., 2006; Lee et al., 2006; Saetrom et al., 2006; Meola et al., 2009; Winter et al., 2009; Ryan et al., 2010). These pri-miRNAs are then cleaved into 60-100 nt, stem loop precursor miRNAs (pre-miRNAs) by Drosha-DGCR8 complex (RNase III endonuclease) (Denli et al., 2004; Du and Zamore, 2005; Kusenda et al., 2006; Zeng, 2006; Kim and Kim, 2007; Reddy et al., 2007; Meola et al., 2009; Winter et al., 2009). The Drosha-DGCR8 complex which is also known as microprocessor complex, recognized and cleaved at the 'ssRNA–dsRNA junction-anchoring' structure which is embedded within pri-miRNA (Figure 1-4) It is a stem of approximately 33 base pairs (bp) with a terminal loop and flanking segment. Drosha-DGCR8 cleaves at ~11 bp from the stem ssRNA junction that is critical for the processing of pre-miRNA (Han *et al*., 2006; Yeom *et al*., 2006; Duan *et al*., 2007). Processed pre-miRNAs are exported out of the nucleus to cytoplasm by RAN-GTPase/exportin-5 (Bartel, 2004; Krol *et al*., 2004; Lee *et al*., 2006; Kim and Nam, 2006; Kim and Kim, 2007; Reddy *et al*., 2007; Winter *et al*., 2009) and succeedingly cleaved by RNase III Dicer to release a double stranded miRNA/miRNA complementary duplex of 18-28 nt, with a 2 nt 3' overhang (Du and Zamore, 2005; Gregory *et al*., 2005; Kim and Nam, 2006; Saetrom *et al*., 2006Reddy *et al*., 2007; Winter *et al*., 2009; Kunej *et al.*, 2012; Slaby *et al.*, 2012). Only mature miRNA will associate with RNA-induced Silencing Complex (RISC) (Gregory *et al*., 2005; Kim and Nam, 2006; Saetrom *et al*., 2006; Zhang *et al*., 2006; Reddy *et al*., 2007; Slaby *et al.*, 2012) or ribonucleoprotein (RNP) complex (Mourelatos *et al*., 2002; Bartel, 2004; Lee *et al*., 2004; Kim and Nam, 2006; Kusenda *et al*., 2006; Reddy *et al*., 2007; Meola *et al*., 2009; Winter *et al*., 2009). While the other miRNA complementary (miRNA*) strand is usually degraded, although recently miRNA* has been found to

play similar role as miRNA (Du and Zamore, 2005; Lee *et al*., 2006**;** Saetrom *et al*., 2006; Meola *et al*., 2009; Slaby *et al*., 2012).

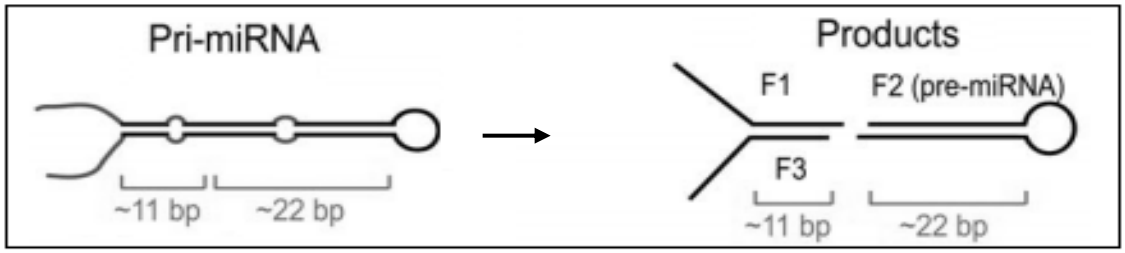


Figure 1-4: The 'ssRNA–dsRNA junction-anchoring' model for pri-miRNA processing. (Figure from Han *et al*., 2006)

This miRNA-protein complex is responsible for the post-transcriptional regulation at transcription level or/and translation level. MicroRNA mediates degradation based on its base pair complementary to its 3' UTR mRNA target: partial complement will result in inhibition of protein translation and 3' UTR mRNA target cleavage for those who have perfect complementary (Ambros, 2004; Bartel, 2004; Kim and Nam, 2006; Zambon *et al*., 2006; Reddy *et al*., 2007; Meola *et al*., 2009; Slaby *et al*., 2012). In the recognition of 3' UTR mRNA target by miRNA, a perfect complementarity is usually restricted to the seed region (2-7 or 2-8 nt of the 5' region of mature miRNA) of the 3' region of the untranslated region of mRNA target (Figure 1-5) (Du and Zamore, 2005; Kim and Nam, 2006; Meola *et al*., 2009; Winter *et al*., 2009).

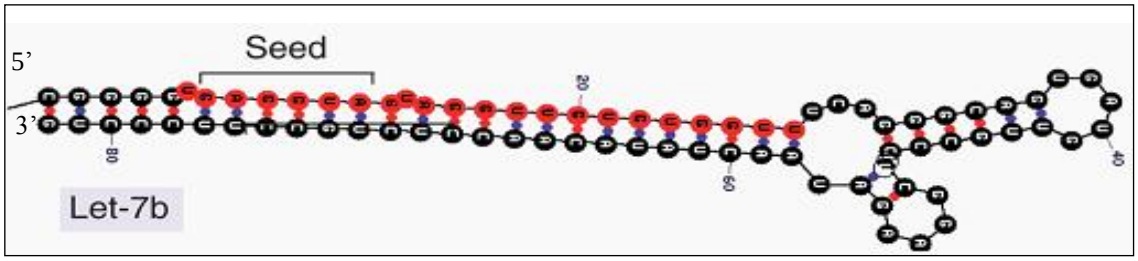


Figure 1-5: Secondary structure of Let-7b.
Red circles are the mature miRNA sequence of Let-7b; seed region is located the 2nd to 8th nt of 5' end of mature miRNA. (Figure from Mishra and Bertino, 2009)

1.2.2.3 Biological functions of human microRNA

At present, many reports indicate that a single miRNA is able to repress a large set of 3' UTR targets, resulting in low protein expression levels within the miRNA-expressing cells (Ambros, 2004; Bartel, 2004; Kim and Nam, 2006; Kusenda *et al*., 2006; Zhang *et al*., 2006; Meola *et al*., 2009). Example in *C. elegans*, absent of *let-7* causes failure in transition of larva to adult (Hammond, 2006; Kusenda *et al*., 2006). In addition, miRNAs are known to involve in cell proliferation and apoptosis, as they regulate pathways that are controlled by *p53*, MYC and RAS genes (Lai, 2003; Ambros, 2004; Bartel, 2004; Hammond, 2006; Zhang *et al*., 2006; Cho, 2007). For instance, some miRNA clusters have been shown to act as a functional switch between proliferation and apoptosis of cell (Plasterk, 2006; Treiber *et al.*, 2012). Besides, miRNAs play role in the determination of timing and maintenance of tissue or cell identity. High miRNA expression level is found in certain tissue or cell type or at particular developmental stage (Zeng and Cullen, 2003; Ambros, 2004; Bartel, 2004; Du and Zamore, 2005; Zhang *et al*., 2006). These small miRNA also take place in responding to environmental stresses, immune system, and neurological processes besides playing an important role in biological processes (Zeng and Cullen, 2003; Bartel, 2004; Du and Zamore, 2005; Kusenda *et al*., 2006; Zhang *et al*., 2006; Wiemer, 2007; Garofalo *et al*., 2008; Meola *et al*., 2009; Treiber *et al.*, 2012).

1.2.3 Type of genetic variations in microRNA

MicroRNA sequences are known to be well conserved, but variations do take place in miRNA with low frequency (Lai, 2003; Saunders *et al*., 2007; Yu *et al*., 2007; Chen *et al*., 2008). Many miRNA variant may not have significant phenotypes as several different miRNAs are able to target the same 3' UTR mRNA transcript where miRNAs would compensate for each other. Moreover, most of the predicted single

nucleotide variant within seed region of mature miRNA sequence are likely neutral or in a mutation selection balance (Du and Zamore, 2005; Kim and Nam, 2006; Lee *et al*., 2006; Zhang *et al*., 2006; Borel and Antonarakis, 2008). Not much miRNA variants are discovered since the rare or common genomic variant are not fully identified. After miRNAs are better characterized and more public available genomic variations database, more studies on miRNA variant can be done in the near future (Borel and Antonarakis, 2008; Lai, 2003; Saunders *et al*., 2007; Yu *et al*., 2007; Chen *et al*., 2008).

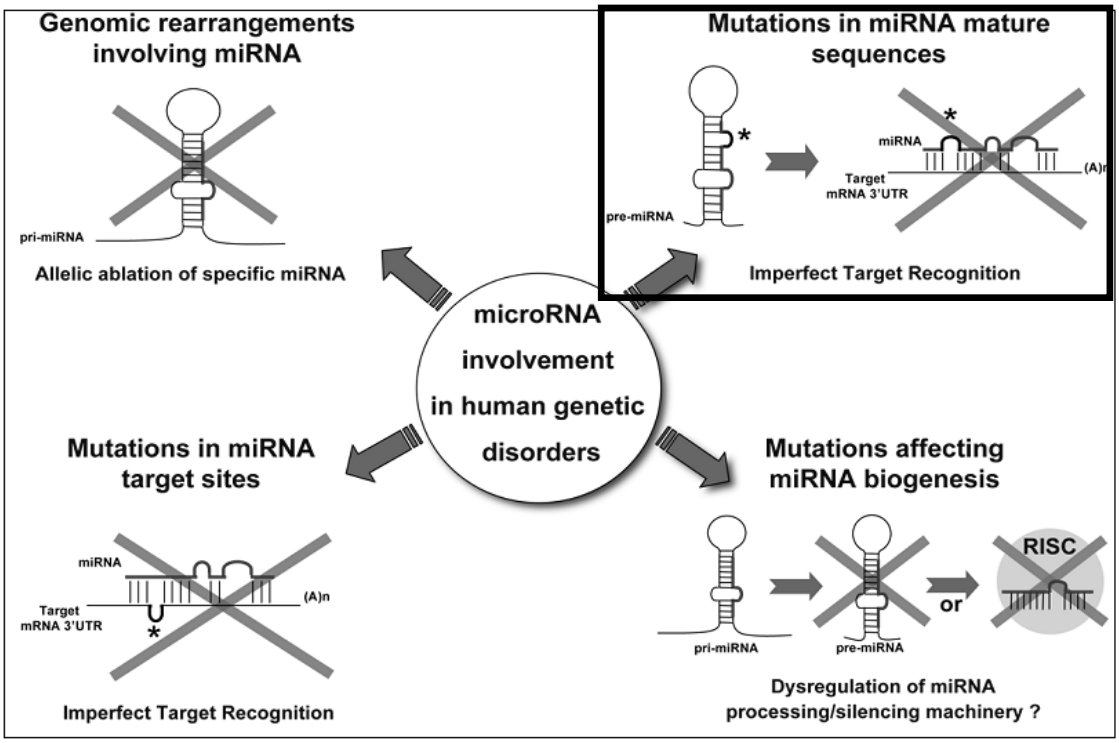


Figure 1-6: Summary of types of miRNAvariant. (Figure from Meola *et al*., 2009)

There are four main types of sequence variations (Figure 1-6) that may affect miRNA function: variation within pri-miRNA or pre-miRNA sequences (Iwai and Naraba, 2005; Diederichs and Haber, 2006; Wu *et al*., 2008), variations in mature miRNA sequence (Iwai and Naraba, 2005; Duan *et al*., 2007; Borel and Antonarakis, 2008; Mencìa *et al*., 2009), variations in the binding target site of 3' UTR mRNA (Abelson *et al*., 2005 ; Saunders *et al*., 2007; Yu *et al*., 2007; Chen *et al*., 2008; Shen *et*

al., 2008), and variations in genes or proteins that are involved in miRNA processing and regulation (Diederichs and Haber, 2007; Yu *et al*., 2007; Meola *et al*., 2009; Boominathan, 2010). In this study, variations in mature miRNA sequence are emphasized.

1.2.3.1 Genetic variations in mature microRNA sequences

Point mutations within the mature miRNA sequences are known to be causal factors of diseases (Iwai and Naraba, 2005; Duan *et al*., 2007; Borel and Antonarakis, 2008; Wu *et al*., 2008; Mencìa *et al*., 2009; Meola *et al*., 2009). Variation within mature miRNA was first discovered to have extensive biological effect by Duan and colleagues (2007). They worked on variation in miRNA, in particular a single nucleotide polymorphism (SNP), rs12975333, at the 8^{th} nt of the 5' end of mature miR-125a. This variation on mature miR-125a had lowered the expression of miR-125a. Down-regulation of miR-125a is suspected to play an important role in the pathogenesis of breast cancer and non-small cell lung cancer (Duan *et al*., 2007; Borel and Antonarakis, 2008; Jiang *et al*., 2010; Mencìa *et al*., 2009; Meola *et al*., 2009). In addition, single nucleotide variation in human mature miR-96 (a single base change A>T in the seed region of 5'-end) was the first example of miRNA implicating human Mendelian disorder (Mencìa *et al*., 2009; Meola *et al*., 2009). However, the occurrence of variations in functional seed region is less than 1% which is relatively rare, as the seed region is well conserved (Saunders *et al*., 2007; Yu *et al*., 2007; Chen *et al*., 2008).

Hypothetically, variation within mature miRNA sequence causes pri-miRNA unable to process into pre-miRNA normally, thus destabilize the interaction with 3' UTR mRNA targets and hence reduced the efficiency of mRNA target inhibition (Duan *et al*., 2007; Georges *et al*., 2007; Borel and Antonarakis, 2008; Horikawa *et al*., 2008;

Wu *et al*., 2008; Yu *et al*., 2008; Mencìa *et al*., 2009; Meola *et al*., 2009). Few studies also stated that variation in mature miRNA may potentially alter the normal biological processes by either changing the processing or target recognition of miRNAs (Borel and Antonarakis, 2008; Wu *et al*., 2008; Mencìa *et al*., 2009; Meola *et al*., 2009; Slaby *et al.*, 2012). The pri-miRNA processing is an important and critical step in miRNA biogenesis, as it determines the sequence of the mature miRNA. Therefore any alteration in the miRNA processing is believed to affect the expression level of mature miRNAs (Borel and Antonarakis, 2008; Wu *et al*., 2008; Mencìa *et al*., 2009; Slaby *et al.*, 2012). Consequently, this may affect the function of miRNA in miRNA-mRNA target interaction. Therefore one small sequence variation at the mature miRNA-binding sites would not only affect the interaction of miRNA and mRNA, but also has a huge effect on hundreds of their miRNA targets (Duan *et al*., 2007; Yu *et al*., 2007; Borel and Antonarakis, 2008; Wu *et al*., 2008; Mencìa *et al*., 2009; Meola *et al*., 2009). If the miRNA targets are oncogene or tumour suppressor genes, these genes might contribute to the development of cancers (Diederichs and Haber, 2006; Yu *et al*., 2007; Wu *et al*., 2008; Kunej *et al.*, 2012; Slaby *et al.*, 2012).

1.2.3.2 Relationship of microRNA to diseases and cancer

miRNA has been found to play a critical role in the development of human diseases and cancer (Kusenda *et al*., 2006; Garofalo *et al*., 2008; Sassen *et al*., 2008; Sethupathy and Collin, 2008; Meola *et al*., 2009; Slaby *et al.*, 2012). Defection in miRNA functions or aberrant miRNA expression level may lead to abnormal miRNA biogenesis and eventually cause disorder or diseases (Kusenda *et al*., 2006; Garofalo *et al*., 2008; Sassen *et al*., 2008; Sethupathy and Collin, 2008; Meola *et al*., 2009). This can be caused by either *cis* factors (chromosome alterations, epigenetic modifications, mutation of promoter element and polymorphisms within miRNA sequences) or *trans*

factors (polymorphisms in miRNA target sites and functional mutation in proteins that involved in miRNA transcription, processing and targeting) (Sethupathy and Collin, 2008).

Currently, numerous evidences suggest that human diseases are correlated with presence of miRNA. In Alzheimer's disease, miR-107 was significantly down regulated and miR-125b was upregulated in brain (Garofalo *et al*., 2008; Nelson *et al*., 2008; Patel *et al*., 2008; Martino *et al*., 2009; Zeng, 2009). Similarly, in Parkinson patient, their midbrain show very low expression level of miR-133b compare to the normal human (Garofalo *et al*., 2008; Nelson *et al*., 2008; Martino *et al*., 2009; Zeng, 2009). MicroRNAs have been shown to regulate genes that are involved in cardiac function and heart development. miR-1 is over-expressed in individuals with coronary artery disease (Garcia and miska, 2005; Rooij *et al*., 2006; Wiemer, 2007; Garofalo *et al*., 2008; Meola *et al*., 2009). Moreover, miRNA expression levels also greatly differ in fragile X syndrome (Kusenda *et al*., 2006; Garofalo *et al*., 2008; Dai *et al*., 2009), Tourette's syndrome (Wiemer, 2007), schizophrenia (Garcia and miska, 2005; Garofalo *et al*., 2008; Feng *et al*., 2009), and Duchenne muscular dystrophy (Wiemer, 2007; Garofalo *et al*., 2008).

Meanwhile, significant increase or decrease of miRNA expression level may change the pathways that are involved in tumour cell proliferation, and hence promote cancer progression and/or tumour formation (Garcia and miska, 2005; Gregory and Shiekhattar, 2005; Garzon *et al*., 2006; Gaur *et al*., 2007; Winter *et al*., 2009; Kunej *et al*., 2012). In cancer, miRNAs that target oncogenes are known to act as tumour suppressors; miRNAs that target tumour suppressors have a role as oncogene (Garcia and miska, 2005; Garzon *et al*., 2006; Zhang *et al*., 2007). For example, miR-15a and

miR-16-1 in chronic lymphocytic leukaemia, and let-7 in lung cancer and non-small cell lung cancer are found to have tumour suppressing activity (Garcia and miska, 2005; Garzon *et al*., 2006; Hammond, 2006; Kusenda *et al*., 2006; Cho, 2007; Pan *et al*., 2007; Garofalo *et al*., 2008; Xia, 2008) while miR-372 and miR-373 in testicular germ cell tumour, miR-21 in breast cancer and miR-155 in Hodgkin's Burkitt lymphoma have oncogenic potential (Garcia and miska, 2005; Garzon *et al*., 2006; Cho, 2007; Pan *et al*., 2007; Garofalo *et al*., 2008; Xia, 2008).

1.2.4 Future prospects of microRNA

The discovery and understanding of miRNA roles in various biological and pathological processes will offer many possible therapeutic applications. The concept of one miRNA modulates hundreds to thousands of mRNAs will provide new window for diagnostics and prognostics therapy of many human diseases, including cancer (Du and Zamore, 2005; Cho, 2007; Garzon *et al*., 2006; Kim and Nam, 2006; Kusenda *et al*., 2006; Lee *et al*., 2006; Meola *et al*., 2009; Zhang *et al*., 2006; Garofalo *et al*., 2008). MicroRNA with different expression profile can be used as biomarkers to detect and classify diseases or tumors according to the differentiation and developmental states (Garcia and miska, 2005; Gregory and Shiekhattar, 2005; Garzon *et al*., 2006; Zhang *et al*., 2007). In addition, miRNA itself can function as tumour suppressors to prevent or cure diseases (Garcia and miska, 2005; Hammond, 2006; Cho, 2007). Future therapies could be the innovation of mimicking or antagonizing miRNA action on multiple targets with small molecules of artificial miRNA or anti-miRNA, to treat diseases or cancers that are difficult to cure (Garcia and miska, 2005; Zhang *et al*., 2007; Garofalo *et al*., 2008).

1.3 Objectives

MicroRNAs are important regulators of development, cell signaling, stress responses, and involved in antiviral defences (Zeng and Cullen, 2003; Bartel, 2004; Du and Zamore, 2005; Kusenda *et al.*, 2006; Kunej *et al.*, 2012; Slaby *et al.*, 2012). Sequence variation in miRNA gene has potention contribution to human disease pathogenesis. In this study, I hypothesized that single nucleotide variant in the seed-regions of mature miRNA might altered the biogenesis of miRNA, therefore up-regulation or down-regulation of mature miRNA expression level, might affects the stable processing of mature miRNA. Hence this would contribute to the pathogenesis of human diseases (Kusenda *et al*., 2006; Garofalo *et al*., 2008; Sassen *et al*., 2008; Sethupathy and Collin, 2008; Meola *et al*., 2009).

Therefore this study aims to achieve the following:

1. To identify single nucleotide variant within the seed region sequence of mature miRNAs by screening single nucleotide polymorphisms databases (dbSNP) against miRNA databases.
2. To study the effects of single nucleotide variant within seed region of mature miRNA sequence toward miRNA bio-processing.

Discovery of single nucleotide variant within seed region of mature miRNA sequence gives a great opportunity to explore the potential connection between variant miRNA genes and their 3' UTR mRNA targets (Garcia and miska, 2005; Zhang *et al*., 2007; Garofalo *et al*., 2008). This study would provide new insights into pharmaceutical research and therapeutic interventions.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Component of PCR:

10X Optimized DyNAzyme™ Buffer [10 mM Tris (pH 8.8)

50 M KCl, 1.5 mM $MgCl_2$

0.1 % Triton® X-100]

0.2 mM of deoxyribonucleotide triphosphates (dNTP)

0.33 µM of forward (F1) and reverse primers (R1)

0.03 U of DyNAzyme™ II DNA polymerase

Component of Cloning and Transformation:

Vector: pcDNA™ 3.3-TOPO® TA (Invitrogen) for miR-125a

PCR 4-TOPO vector (Invitrogen) for miR-124-3

pGEM®-T Easy Vector Systems (Promega) for miR-662

Salt solution (200 mM NaCl, 10 mM $MgCl_2$)

One Shot® TOP 10 *E.coli* competent cells

S.O.C. medium (Super Optimal broth with catabolic repression)

Ampicillin (100 µg/ml)

Component of Ligation:

pcDNA ™ 3.1/Zeo (-) vector (Invitrogen)

pcDNA™ 3.3-TOPO® TA vector (miR-125a); PCR 4-TOPO (miR-124-3) and pGEM®-T Easy vectors(miR-662)

Restriction enzyme: *Hind*III, *Xba*I and *Eco*RI

T4 DNA Ligase

T4 DNA Ligase Reaction Buffer [50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP and 10 mM Dithiothreitol (pH 7.5)]

Component of Mutagenesis:

10X PCR Buffer (300 mM Tris [pH 9.0]

200 mM salts consisting of Na^+ and $NH4^{2+}$ and 20 mM Mg^{2+})

0.12 mM of deoxyribonucleotide triphosphates (dNTP)

0.3 µM of each mutagenic forward and reverse primer

0.05 U of i-pfu DNA polymerase (iNtRON)

T4 DNA Ligase and Reaction Buffer [50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP and 10 mM Dithiothreitol (pH 7.5)]

Component of Cell Culture:

Human Embryonic Kidney 293T (HEK293T) adherent cells

DMEM-5 (Dulbecco's Modified Eagle Medium)

5% (v/v) fetal bovine serum (FBS)

100 µg/ml penicillin/streptomycin

Component of HEK293T Cell Transfection:

Green Fluorescent Protein control vector, pEGFP (Clontech)

pcDNA™ 3.1 plasmid : wild-type allele of pri-miRNAs and pri-miRNA variant

Opti-MEM I Reduced Serum Medium (serum-free and without antibiotics)

Fugene® HD Transfection Reagent (Roche)

Component of Total RNA Extraction:

Extraction buffer [4M of guanidine isothiocyanate, 0.02M of sodium citrate, 0.5% of sarcosyl and 0.1M of mercaptoethanol]

2M sodium acetate (pH 4)

Acidic phenol

chloroform:isoamylalcohol (24:1)

isopropanol

DEPC-treated water

DNase enzyme and buffer

Component of cDNA synthesis:

(a) Reverse transcription (RT) reaction for pri-miRNA and pre-miRNA :

1 X RT Buffer

4 mM of 25 X dNTP mix

1 X RT random primers

2.5 U of MultiScribe™ Reverse Transcriptase

(b) Reverse transcription (RT) reaction for mature miRNA :

1 X RT buffer

1 mM dNTP mix

0.25 U RNase inhibitor

3.33 U of MultiScribe™ Reverse Transcriptase

3 µl of 5 X TaqMan® MicroRNA RT primer

Component of TaqMan miRNA Real Time PCR:

1 X TaqMan® Fast Advanced Master Mix (2X)

1 X of TaqMan or Custom TaqMan Gene Expression Assay (20X)

Component of SYBR Green Real Time PCR:

1 X of Power SYBR Green PCR Master Mix (2X) [SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase LD, dNTPs with dUTP/dTTP blend, Passive Reference 1 and optimized buffer component]

50 nM of forward and reverse primers

2.2 Methods

2.2.1 MicroRNA database

During this study was designed, the published sequences of *Homo sapiens* miRNAs were downloaded from miRBase Registry, Trust Sanger Institute (http://microrna.sanger.ac.uk/sequences/; Release 16.0, September 2010) (Jones, 2004; Jones *et al*, 2006; Jones *et al*, 2008). A total of 1048 hairpin pre-miRNA sequences of *Home sapiens* were obtained. All the sequences of pre-miRNA contained sequence of mature miRNAs. Within these 1048 hairpin precursor entries, there are 1344 of mature miRNA sequences. This is because some hairpin precursors may contain two functional mature miRNAs. The chromosomal coordinates for these 1046 pre-miRNAs were also obtained from miRBase Registry version 16.0.

2.2.2 Local Alignment of miRNA and SNP databases

Local alignment between hairpin pre-miRNA sequences and a wide range of single nucleotide polymorphisms (SNP) sequences in SNP database were carried out using BLAT (Basic Local Alignment Search Tool - Like Alignment Tool) (Kent, 2002). BLAT is available via web interface at UCSC Genome browser (http://genome.ucsc.edu/). SNP databases used were dbSNP version 131 and 132. BLAT program is able to scan relatively short matches rapidly, and extends into high-

scoring pairs. Parameters used were by default and assembly chosen is "Feb. 2009 (GRCh37/hg19)".

From the BLAT search results, the highest score and identity of near-perfect match sequences were chosen. Variant allele of SNP sequence that falls on the pre-miRNA sequences were chosen. Type of variant allele on pre-miRNA and mature miRNA sequences were recorded. Single nucleotide variant within seed region sequence of mature miRNAs were chosen and recorded. The genomic coordinates of these selected miRNAs were confirmed to match with the related SNPs. The potential single nucleotide variant within seed region sequence of mature miRNAs was used as a template for the secondary structures prediction by using MFOLD program (section 2.2.3). Figure 2-1 shows the flow of selecting potential single nucleotide variant within the seed region sequence of mature miRNAs.

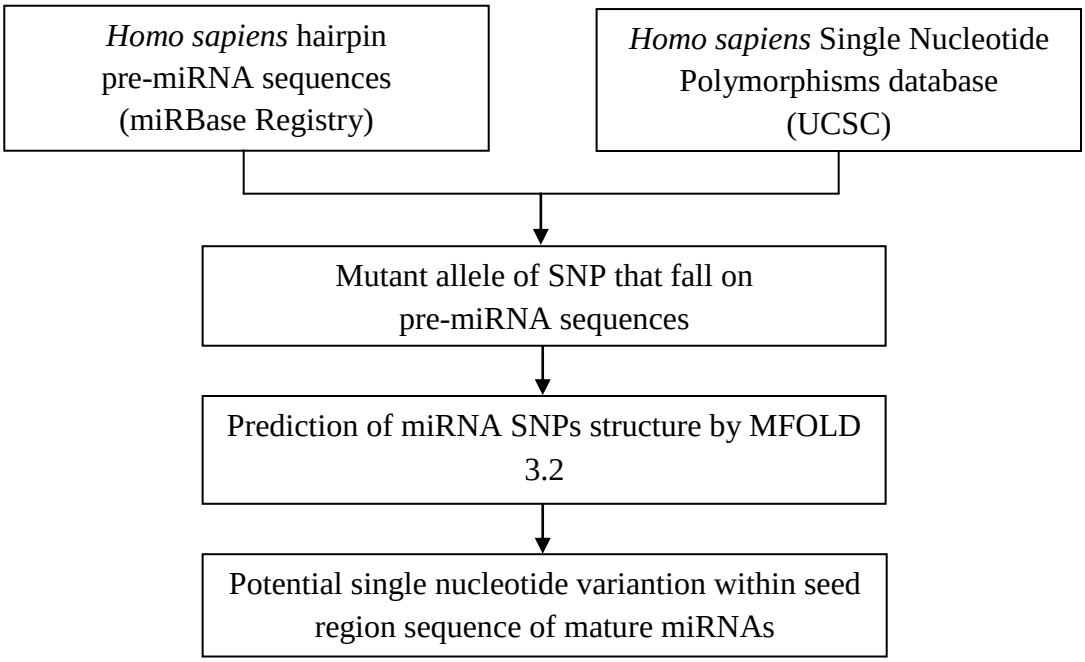


Figure 2-1: Flowchart for bioinformatics approaches to identify potential single nucleotide variation within seed region sequence of mature miRNAs.

2.2.3 Prediction of potential single nucleotide variant within pre-miRNAs using secondary RNA structures

The selected pre-miRNAs from BLAT analysis were subjected to secondary structure prediction using a publicly available web-based computational software MFOLD version 3.2 (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). The outputs of MFOLD include minimum free energy (MFE), the number of nucleotides, location of the matched region, and the number of arms per structure (Zhang et al., 2007). Both wild-type and single nucleotide variant of the same pre-miRNAs were deposited into the software for prediction of the most stable secondary structure. The minimal folding energy (MFE), expressed in kcal/mol, is a method of calculating the thermodynamic stability of the secondary structure of RNA. A stable secondary structure of pre-miRNA has a high negative the minimal folding energy (MFE). Minimal folding free energies of pre-miRNAs were estimated using the program MFOLD with default parameter values (Ambros *et al*., 2003; Zuker, 2003; Bonnet *et al*., 2004; Zhang *et al*., 2006; Zhang *et al*., 2007). The suitable secondary structure outputs were first selected based on the high negative minimal folding energy.

Theoretically, change of one nucleotide may alter the secondary structure of the wild-type allele of pre-miRNA transcript. Single nucleotide variant within pre-miRNA sequence that has secondary structure with base pair mismatch (guanine–uracil pairing), decreased minimal free energy values or enlarged RNA bulges at the seed region of mature miRNA will first be selected. These conformation changes may alter the processing of pre-miRNA into functional mature miRNA and hence affect the binding of mature miRNA to 3’ UTR target genes. For the single nucleotide variant within seed region of mature miRNA sequence that did not affect hydrogen bonds or minimal free energy of the predicted secondary structure were excluded from this study.

2.2.4 Construction of DNA plasmid

2.2.4.1 Genomic DNA extraction from blood

Positive control (blood sample of *Homo sapiens*) was provided by NPC lab (University of Malaya). Normal human genomic DNA was isolated by using conventional phenol-chloroform method. Plasma was separated from the whole blood by centrifuging at 402 X *g* for 5 minutes at room temperature. Approximately 8 ml of 1X RCLB (Red Cell Lysis Buffer: 10 mM Tris-HCL [pH 8.0], 5 mM MgCl2, 0.32M sucorse and 0.75% Triton X-100) was added to 2ml of blood and centrifuged at 2,191 X *g* for 10 minutes at 10°C. This step is to lyse the erythrocytes. The pellet was resuspended in 4 ml of RCLB and centrifugation with the same condition was repeated. If the red cells persisted, pellet was washed again. Tubes containing the pellet was air-dried. After the pellet had dried, 80µl of 10 X Proteinase K buffer, 20µl of 20mg/ml Proteinase K, 40µl of 20% Sodium Dodecyl Sulfate (SDS) solution and 400µl of distilled water were added to the pellet. The mixture was incubated overnight at 37°C waterbath. On the second day, tube containing pellet mixture was removed from waterbath and cooled to room temperature before proceeding. Then, 200µl of 6M sodium chloride (NaCl) was added and vortexed vigorously for 2-3 minutes. All the mixture solution was transferred into a new tube and equal amount of phenol-chloroform was added. The solution was centrifugated at 14,400*g* for 30 minutes at 10°C, the clear aqeuous supernatant was transferred to a new tube and 900µl of chilled absolute ethanol was added. Mixture was incubated at -20°C for 1 hour. Later, centrifugation at 14,400*g* for 5 minutes at 4°C was performed to remove the absolute ethanol. The pellet was washed with 1 ml of 70% ethanol and the ethanol was removed after centrifugation at 14,400g for 5 minutes at 4 °C. After that, the pellet was resuspended in 100µl of TE buffer [10mM Tris (pH 8.0), 1mM EDTA].

2.2.4.2 Agarose gel electrophoresis

The quality of the genomic DNA was inspected by agarsoe gel electrophoresis. Approximately 5µl of genomic DNA and 1µl of loading DNA dye per well were loaded onto 0.8% agarose gel (0.16g agarose and 20ml of Tris-Borate-EDTA [TBE buffer: Tris base, boric acid and 0.05M EDTA with pH 8.0]). The gel was run at 150V for 40 min, in 1 X TBE buffer.

2.2.4.3 Quantification and quality of genomic DNA

Quantification of DNA was done in a UV Spectrophotometer with the absorbance at 260 and 280nm. Protein shows optimal absorbance at 280nm wavelength, thereby quantitate the amount of contaminating protein present in the sample. Therefore, the ratio of the absorbance at 260nm/280nm indicates the purity of DNA sample. The acceptable range is from 1.7 to 1.9.

2.2.4.4 Primer design for Polymerase Chain Reaction (PCR) and mutagenesis

Primers that annealled pri-miRNA were used to amplify pri-miRNA from human genomic DNA by using PCR. Although the length of pri-miRNA varies significantly, approximately 100bp flanking sequences on either side of pre-miRNA is sufficient for miRNA biogenesis (Duan *et al*., 2007). Thus, focus was given on approximately 450bp that covers the mature miRNA and 200bp of flanking sequences on each side.

In this study, two pairs of forward and reverse PCR primers (Table 2.1) were designed: miR-124-3 and miR-662 and miR-125a. The primers were designed with the aid of "FastPCR" and "BioEdit" software that were downloaded from www.biocenter.helsinki.fi/bi/Programs/download.htm and www.mbio.ncsu.edu

respectively. Besides, two pairs of primers for single nucleotide variation were designed for each mature single nucleotide variation within seed region of mature miRNA sequence (Table 2.2). The desired mutant nucleotide was placed in the centre of both forward and reverse mutagenic primers. Stratagene's web-based — QuikChange ® Primer Design Program, a publicly available web-based was used (http://www.stratagene.com/qcprimerdesign).

Table 2.1: Primers sequences for PCR: ranging from 20-22nt; 50-60% of GC content and melting temperature within 55-58°C.

Applications	Primer	Sequences
Polymerase chain reaction (PCR) : recognized pri-miRNA	miR125a-wF	5'-CAC AGT GGA TCC TCT GAC TCC-3'
	miR125a-wR	5'-CCA TCG TGT GGG TCT CAA GG-3'
	miR124-wF	5'-AAA GGG GAG AAG TGT GGG CTC-3'
	miR124-wR	5'-GCA TTG TTC GCC GGA TTT GTC C-3'
	miR662-wF	5'-ATA CCT GAG GTG GAG GCC TG -3'
	miR662-wR	5'-CAC AGG TCA CAG CCA GCA TAC C-3'

Table 2.2: Primers sequences for mutagenesis: ranging from 30-45nt, at least 40% of GC content and melting temperature greater or equal to 78°C .

Applications	Primer	Sequences
Site-directed mutagenesis	miR125a-mF	5'-GCC AGT CTC TAG GTC CCT GA**T** ACC CTT TAA CC-3'
	miR125a-mR	5'-GGT TAA AGG GT**A** TCA GGG ACC TAG AGA CTG GC -3'
	miR124-mF	5'-GGA CCT TGA TTT AAT GTC TAT ACA ATT AA**T** GCA CGC GGT GAA TGC-3'
	miR124-mR	5'-GCA TTC ACC GCG TGC **A**TT AAT TGT ATA GAC ATT AAA TCA AGG TCC-3'
	miR662-mF	5'-CTG AAG GTC TCC CAC **A**TT GTG GCC CAG CAG-3'
	miR662-mR	5'-CTG CTG GGC CAC AA**T** GTG GGA GAC CTT CAG-3'

2.2.4.5 Polymerase chain reaction (PCR) amplification

Two pri-miRNA sequences were first amplified from genomic DNA (blood provided from NPC lab) by using PCR. Each amplification reactions was performed in a 15µl volume that contained 10X Optimized DyNAzyme^{TM} Buffer [10mM Tris (pH 8.8), 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton® X-100], 0.2mM of deoxyribonucleotide triphosphates (dNTP), 0.33µM of forward (F1) and reverse primers (R1), and 0.03U of DyNAzyme^{TM} II DNA polymerase (Finnzymes). Extracted genomic DNA (20ng/µl) was added and PCR was performed in Veriti Thermal Cyler (Applied Biosystems). The amplification was repeated for 35 cycles: denaturation for 30s at 94°C, primer annealing for 30s and enzymatic chain extension for 40s at 72°C, followed by a single terminal extension at 72°C for 5 min. Distilled water was used a template as negative control. The fragment sizes of PCR products were visualized with ethidium bromide followed by gel electrophoresis (as mentioned in section 2.4.2). Agarose gel of 1.5% was prepared. VC 100 bp Plus DNA ladder (Vivantis) was used to compare the fragmented size.

2.2.4.6 Purification of PCR products

Polymerase chain reaction products were purified using PureLink^{TM} Gel Extraction Kit (Invitrogen). PCR product purification was carried out following manufacturer's instructions. Briefly, the DNA fragment of interest were excised from the agarose gel, transferred into microcentrifuge tubes resuspended in Gel Solubilization buffer. The mixture was incubated at 50 ºC for 15 minutes until the gel was completely dissolved. The dissolved gel slice was transferred to a spin column and centrifuged at 10,500 *g* for 1 min. The flow-through in the collection tube was discarded and washed with Washing Buffer added to the column and centrifuged at 10500 *g* for 1 min. Flow-through was again discarded, the spin column dried and placed in a 1.5ml

microcentrifuge tube. Purified DNA was eluted with 30µl of nuclease free water, incubated at room temperature for 2 min and centrifuged for 1 min to collect the DNA The microcentrifuge tubes containing eluted DNA was stored at -20ºC. Gel electrophoresis (as section 2.4.2) was run to confirm the size of DNA obtained.

2.2.4.7 Cloning and transformation of pri-microRNA

Purified DNA fragment of pri-miRNAs were cloned into different vectors: pcDNA™ 3.3-TOPO® TA (Invitrogen) for miR-125a, PCR 4-TOPO vector (Invitrogen) for miR-124-3 and pGEM®-T Easy Vector Systems (Promega) for miR-662. A mixture of 4µl of fresh PCR product, 1µl of TOPO® or pGEM®-T Easy vector and 1µl of salt solution (200 mM NaCl, 10 mM $MgCl_2$) was added. The TOPO cloning reaction was incubated at room temperature for 15 minutes, while pGEM®-T Easy cloning reaction was incubated at 4ºC overnight. One Shot® TOP 10 *E.coli* competent cell is used as a host for transformation. Incubation on ice for 5 minutes was performed after 5µl of cloning reaction was added into 50µl of chemically competent cell. Plasmid was introduced by heat shock for 30 seconds at 42ºC and the competent cells were immediately transferred to ice. Then S.O.C. medium (Super Optimal broth with catabolic repression) was added and the transformants were incubated in shaker incubator at 37ºC for 1 hour with the speed of 200rpm. Finally transformants were plated on LB agar plates supplemented with ampicillin (100µg/ml) for selection. Presence of plasmid was confirmed by performing colony PCR. The primers used are specific forward primer and reverse primer of vector.

2.2.4.8 DNA Plasmid extraction

Colonies with positive transformant were cultured for 16 hours at 37ºC in 10ml of Luria-Bertani broth containing 100µg/ml of ampicillin. Glycerol stock of 800µl bacterial culture was kept before proceeding to plasmid extraction. Bacterial cells were harvested by centrifugation at 6,000*g* for 15 min at 15ºC. Bacterial cell pellets were re-suspended in 250µl of re-suspension buffer (QIAprep Miniprep, Qiagen). Next, 250µl of lysis buffer was added into the tube and immediately another 300µl of neutralization buffer was added. This mixture was bought for centrifugation at 18,000g for 10 minutes. All clear supernatant was transferred to a QiAprep spin column with the maximum volume of 750µl. Supernatant passes through with the aid of centrifugation at 18,000g for 1 minute. Then, 500µl of binding buffer was added, followed by centrifugation and subsequently 750µl of washing buffer. Washing step was repeated twice and finally eluted with 30µl of nuclease free water. Yields were determined by observing DNA on 0.8% of agarose gel and UV absorbance from a spectrophotometer.

2.2.4.9 DNA sequencing

The cloned DNA fragment from all two plasmid (pcDNA™ 3.3-TOPO® TA for miR-125a, PCR 4-TOPO vector for miR-124-3 and pGEM®-T Easy Vector Systems for miR-662) were sequenced to validate its identity. DNA sequencing was performed using a commercial sequencing service (Next Gene Scientific).

2.2.4.10 Sub-cloning into pcDNA vector

The correct sequence of pri-miRNA was then sub-cloned into a mammalian expression vector, pcDNA ^{TM} 3.1/Zeo (-) vector (Invitrogen). Both PCR 4-TOPO and pGEM®-T Easy vectors were cut with same restriction enzyme, *EcoR*I. Three µg/µl of cut DNA fragment was cloned into 5µg/µl of pcDNA vector with the aid of T4 DNA

Ligase and T4 DNA Ligase Reaction Buffer [50mM Tris-HCl, 10mM $MgCl_2$, 1mM ATP and 10mM Dithiothreitol (pH 7.5)]. This ligation was then transformed into One Shot® TOP 10 *E. coli* competent cell. Transformants with the insert were selected by PCR. Sequencing was used to confirm the orientation of the inserted DNA. Glycerol stock was prepared for clones that contained correct sequence of each wild-type allele of pri-miRNA.

2.2.4.11 Mutagenesis

Mutagenesis was performed by amplifying the entire plasmid with primers that contained the desired single nucleotide mutation to the seed region of 5'-end mature miRNA sequence (Figure 2-2). Red bar represent the mutant site in the plasmid.

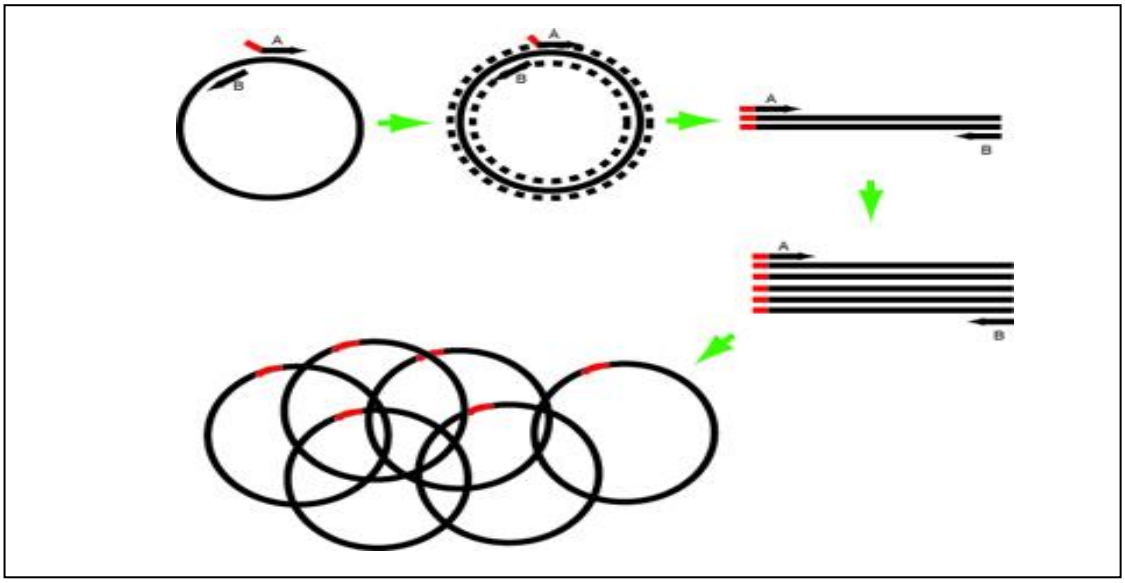


Figure 2-2: Primers A and B contained desire single nucleotide mutation were used to amplify the entire plasmid.
(Figure from http://openwetware.org/wiki/Image:SDMHorn1a.tif)

The recipe of mutagenesis reactions in a volume of 25µl were 10X PCR Buffer (300mM Tris [pH 9.0], 200mM salts consisting of Na^+ and $NH4^{2+}$ and 20mM Mg^{2+}), 0.12mM of deoxyribonucleotide triphosphates (dNTP), 0.3µM of each mutagenic

forward and reverse primers and 0.05U of i-pfu DNA polymerase (iNtRON). Approximately 50ng/µl clone of pcDNA^{TM} 3.1/Zeo (-) vector that contained correct inserted DNA was added into the mixture for mutagenesis. Amplification was performed at 95ºC for 1 minute as initial denaturation, followed by 25 cycles of denaturation at 93ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 9 minutes. Ligation reaction was performed by adding 4.4µl of PCR product to 0.2µl of T4 DNA Ligase and 0.4µl of T4 DNA Ligase Reaction Buffer [50mM Tris-HCl, 10mM $MgCl_2$, 1mM ATP and 10mM Dithiothreitol (pH 7.5)]. The ligation reaction was incubated overnight at 16ºC. Heat shock transformation was performed and positive transformants were selected through colony PCR and confirmed by sequencing.

2.2.4.12 Sequence alignment

DNA sequences of plasmids for both subcloned pcDNA and mutagenized pcDNA were analysed by alignment with pri-miRNA sequences obtained from miRBase database. The publicly available software MEGA 4 (Molecular Evolutionary Genetics Analysis) was used (http://www.megasoftware.net/mega4/mega.html).

2.2.5 Cell culture and transfection

2.2.5.1 Revival of frozen HEK293T cells

Human Embryonic Kidney 293T (HEK293T) adherent cells were kindly provided by Institute of Medical Research (IMR). HEK293T cells were chosen because of undetectable or very low levels expression of the studied miRNAs. Cryovial containing HEK293T was thawed in a 37ºC water-bath for 2 minutes. Cells were added drop-wise into 8ml of pre-warmed growth medium DMEM-5 (Dulbecco's Modified Eagle Medium, Invitrogen; contains 4,500mg/L D-glucose and L-glutamine) to dilute

the DMSO. This DMEM-5 medium contained 5% (v/v) fetal bovine serum (FBS) and 100µg/ml penicillin/streptomycin. Centrifugation at 110 X *g* for 5 minutes was performed. The cell pellets was resuspended with 5ml of DMED-20 (20% (v/v) FBS and 100µg/ml penicillin/streptomycin) and was added into 25cm^2 tissue culture flask for overnight incubation. Incubation was performed in a humidified 37°C, 5% CO2 incubator.

2.2.5.2 Trypsinizing and subculturing cells

Cells were examined microscopically for healthiness and confluency (80-90%). Cells were detached by trypsin treatment and sub-cultured. All DMEM-20 medium was removed from culture flask with sterile serological pipettes. Pre-warmed trypsin (3 ml) (TrypLE™ Express Stable Trypsin Replacement Enzyme, Invitrogen) was added to cover adherent cell layer in the culture flask. Next, the flask was incubated at humidified 37°C CO2 incubator for 5 minutes. Cell detachment was checked under microscope. Trypsin activity was inhibited by adding 4 ml of DMED-10 medium (10% (v/v) FBS and 100µg/ml penicillin/streptomycin) to the detached cells. The cells were centrifugated at 110 X *g* for 5 minutes to pellet the cells. HEK293T cells were then re-suspended with 1ml of DMEM-10 medium. Re-suspended cells were transferred to new 75cm^2 culture flasks that have been labelled appropriately (cell type, date and passage number). The cells were cultured at 37°C 5% CO2 incubator overnight. After 2 days, several passages were repeated for all confluent secondary cultures by replacing fresh medium.

2.2.5.3 Cell number count

Determining cell seeding density and cell viability is important to ensure consistency and reproducibility of experiment. Dilution was prepared by mixing 50µl of cells and 450µl of trypan blue solution. Cells were allowed to settle for a few seconds before cell count is performed. The cells should be evenly distributed on the counting chamber (Figure 2-3). A hand-held counter was used to count the cells on all five squares.

The number of cells was determined by following calculations:

I : cells/ml = average count per square × dilution factor × 10^4*

II: total cells = cells/ml × total original volume of cell suspension from which sample was taken.

* The number 10^4 is the volume correction factor for the hemacytometer. Each square is 1 × 1 mm with the depth of 0.1 mm.

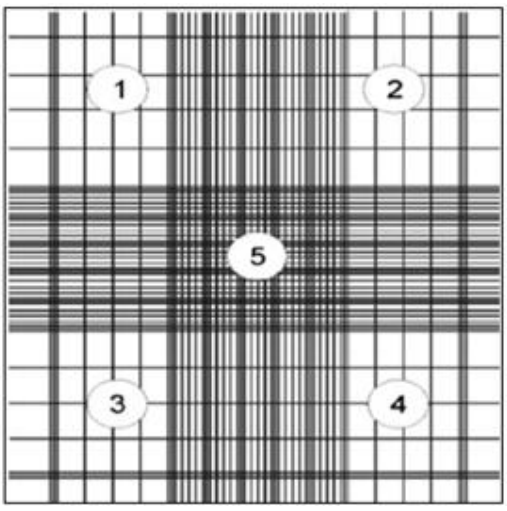


Figure 2-3: Counting chamber with nine squares.
The four corner squares (1, 2, 3, and 4) and the central square (5). (Figure from http://3-b-s.org/cell-count-using-hemocytometer-p-159259.html)

2.2.5.4 Transfection

HEK 293T cells were sub-cultured in 6-well plates at 1 x 10^5 cells/ml the day prior to transfection. Cells were cultured to reach a density of greater than 80% confluency at the time of transfection. The transfection experiment consists of Green Fluorescent Protein control vector - pEGFP (Clontech), an empty vector of pcDNA™ 3.1, three wells containing wild-type allele of pri-miRNA and the remaining three wells contained pri-miRNA variant (Figure 2-4). Transfection complexes were prepared by adding 5µl of Fugene® HD Transfection Reagent (Roche) to 1µg of plasmid DNA that was diluted with Opti-MEM I Reduced Serum Medium (serum-free and without antibiotics), as shown in Table 2.3. The transfection complexes were incubated at room temperature for 15 minutes. Well-mixed transfection complexes were added in a drop-wise manner to all wells that contained cells. Wells were swirl to ensure even distribution of transfection complex. Then, the 6-wells plates were incubated at 37°C in CO_2 incubator for 48 hours. Each transfection was technically repeated three times to check for the reproducibility of the results. pEGFP transfected cells were checked under fluorescence microscope for the consistency and efficiency of transfection.

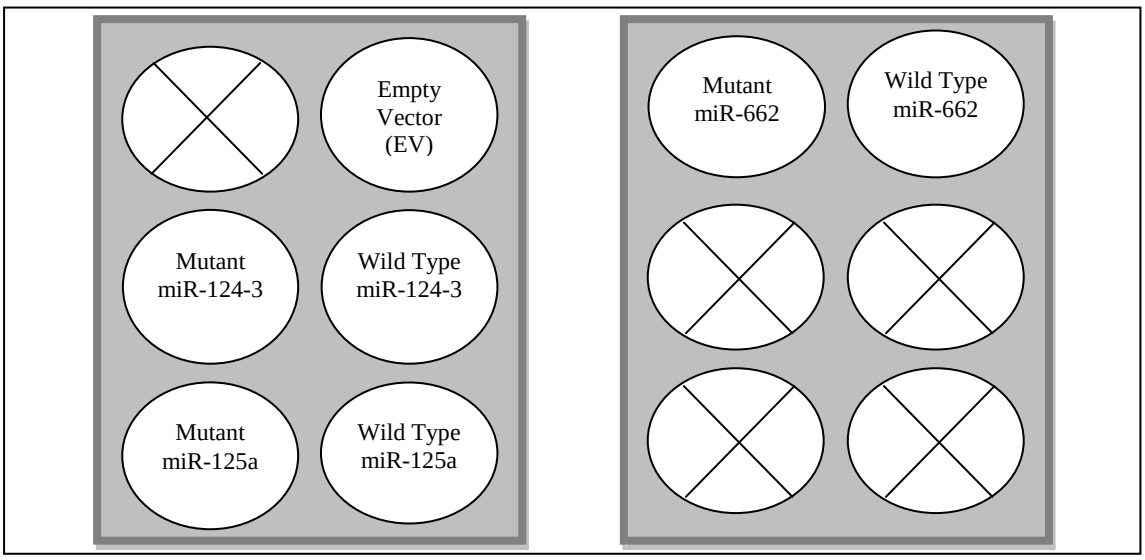


Figure 2-4:Transfection plates consist of one empty vector, three wild-type allele of pri-miRNA, three pri-miRNA variant and five empty wells.

Table 2.3: Recipe for transfection complexes.

Component	1x reaction quantity
DNA	1 µg (4µl)
Fugene	5µl
Buffer	96µl

2.2.6 Quantification experiment

2.2.6.1 Total RNA extraction

Freshly transfected cell cultures were used for total RNA extraction, using the acid guanidine isothiocyanate-phenol: chloroform extraction method (Chomczynski & Sacchi, 1987; Chomczynski & Sacchi, 2006). For HEK293T adherent cells, collection of cells were done by discarding the culture medium. Cells in each well was washed and lysed with 400µl of cold extraction buffer, solution D [4M of guanidine isothiocyanate, 0.02M of sodium citrate, 0.5% of sarcosyl and 0.1M of mercaptoethanol]. Then, lysed cells were transferred from the 6-wells plate to new microcentrifuge tubes. Lysed cells were pre-warmed at 65°C for 10 minutes before adding 40µl of 2M sodium acetate (pH 4). The mixture was mixed vigorously for 20 seconds. A volume of acidic phenol was added and mixed by vortex. Next, 80µl of chloroform: isoamylalcohol (24:1) was added and was mixed well. After the cells were placed on ice for 10 minutes, centrifugation at 16,100*g* for 15 minutes at 6°C was performed. The upper aqueous phase that contained total RNA was carefully transferred to a clean microcentrifuge tubes. The extraction was repeated twice with acidic phenol and centrifuge with the same conditions. A volume of cold isopropanol was added to the clear supernatant. The mixture was incubated at -20°C overnight. On the second day, the tube was centrifuged at 16,100*g* for 30 minutes at 6°C. After that, the supernatant was discarded. The pellet was washed with 75% ethanol and was centrifuged at the speed of 16,100*g* for 10 minutes at 6°C.

This washing step was repeated twice. The cell pellet was allowed to air-dry for 10-15 minutes. Finally the pellet was re-suspended in 40µl of DEPC-treated water and was kept at – 2°C.

Later, the total RNA quality was checked on 1% agarose gel and by NanoDrop spectrophotometer. Values from UV absorbance at 260nm were used to access RNA quantity. The determined absorbance values at 260nm (A_{260}) values were multiplied with the dilution factor and then RNA concentration (µg/ml) can be calculated by equations.

- Total RNA = 40 X A_{260} value

The ratio of the absorbance at 260nm / absorbance at 280nm indicates the purity of RNA sample. The acceptable range is from 1.9 to 2.1.

2.2.6.2 DNase treatment

All extracted total RNA was treated with 1µl of DNase buffer and 1µl of DNase enzyme to remove contaminating genomic DNA. Treated RNA was placed in room temperature for 15 minutes and at 65°C for 20 minutes after adding 1µl of EDTA to stop DNase activity.

2.2.6.3 cDNA synthesis

(a) cDNA for SYBR Green Real Time PCR

Single stranded cDNA was synthesized from DNase treated total RNA by using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). This single stranded cDNA was used as the template to study the expression level of pri-miRNA and pre-miRNA. A volume of 2 µl of reverse transcription (RT) reaction that contained 1 X RT Buffer, 4mM of 25 X dNTP mix, 1 X RT random primers and 2.5U of MultiScribe™

Reverse Transcriptase was prepared. The reverse transcription mixture was mixed well with 2µg of RNA. A thermal cycler was used to perform reverse transcription with the conditions of 1 cycle of incubation at 25°C for 10 minutes, reverse transcription at 37°C for 120 minutes and denaturation of double stranded RNA at 85°C for 5 minutes.

(b) cDNA for Taqman Real Time PCR

To study mature miRNAs that have only about 18-28nt, TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) that uses specific miRNA primers (looped RT primer) was used to synthesis cDNA. This single stranded cDNA is reverse transcribed from the DNAse treated total RNA. Reverse transcription reaction was prepared in a total volume of 15µl that consists of 1 X RT buffer, 1mM dNTP mix, 0.25U RNase inhibitor, 3.33U of MultiScribe™ Reverse Transcriptase and 3µl of 5 X TaqMan® MicroRNA RT primer (Figure 2-5). The input amount of small RNA was 10ng per 15µl RT reaction. Reverse transcription was run with thermal cycler by holding incubation at 16°C for 30 minutes, reverse transcription at 42°C for 30 minutes and denaturation of double stranded RNA at 85°C for 5 minutes. All the newly synthesized cDNAs were kept at 4°C for short term storage or at -20°C for long term storage.

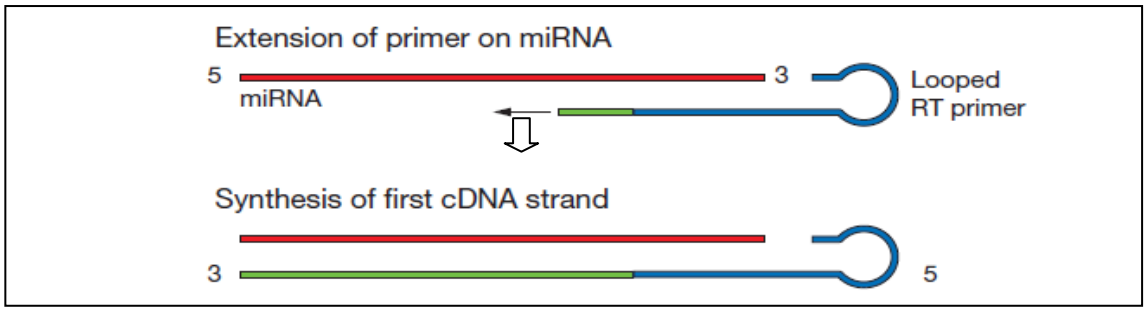


Figure 2-5: Reverse transcription of short single stranded mature miRNA using specific looped RT primer manufactured by Applied Biosystems.
(Figure from http://www3.appliedbiosystems.com/cms/groups/mcb_support/document/generaldocument/cms_042167.pdf)

2.2.6.4 Primer design for SYBR Green Real Time Polymerase Chain Reaction

One pair of specific primer was designed to recognize each pri-miRNA (F1 and R1) and one pair for both pri-miRNA and pre-miRNA (F2 and R2) sequences (Figure 2-6). The sequences of primers for each miRNA sets were shown in Table 2.4. These primers are used for quantification of pri-miRNA and pre-miRNA expression level by using SYBR Green real time PCR (qPCR). These primers were designed using Primer Express® Version 3.0 Software with the following guidelines (primer length is 20 bases, GC content is 30-80% and melting temperature is 58-60°C). The optimal length for amplicons to work efficiently is 50 to 150 base pair.

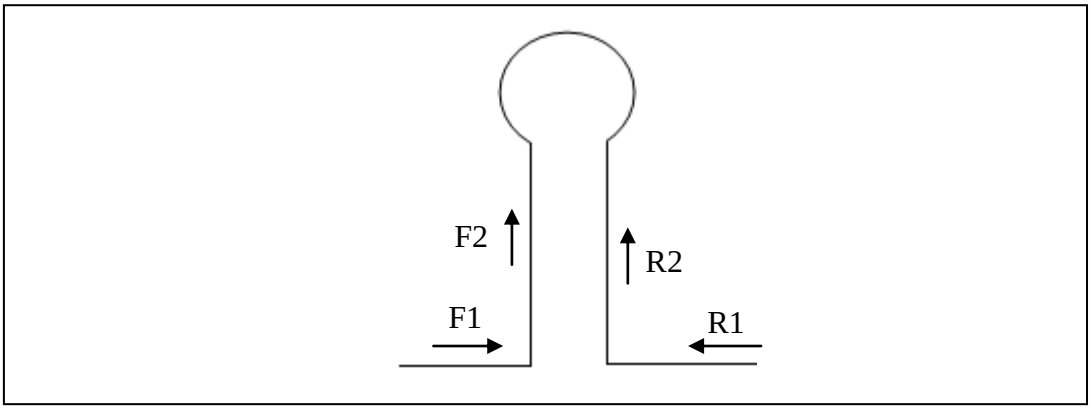


Figure 2-6: The position of primers that recognized pri-miRNA alone (F1 and R1) or both pre-miRNA and pri-miRNA (F2 and R2).

Table 2.4: Primer sequences used in quantitative SYBR Green Real Time PCR.

Pri-miRNA primer F1 and R1	Pri-125a-F1	5'-GCA CAC CAT GTT GCC AGT CT-3'
	Pri-125a-R1	5'-TCC CAA GAA CCT CAC CTG TGA -3'
	Pri-124-F1	5'-TGC GTG TTC ACA GCG GAC C-3'
	Pri-124-R1	5'-ATG AGA AAG GAG CGG CGG-3'
	Pri-662-F1	5'-TAT GCT GGC TGT GAC CTG TGA-3'
	Pri-662-R1	5'-AAA CAA CAG ATG GCT GGC AAC T-3'
Pre-miRNA primer F2 and R2	Pre-125a-F2	5'-TGC CAG TCT CTA GGT CCC TGA-3'
	Pre-125a-R2	5'-GAC GCC AGG TCC CAA GAA CCT -3'
	Pre-124-F2	5'-TGA GGG CCC CTC TGC GTG TTC AC-3'
	Pre-124-R2	5'-AGG CGC CTC TCT TGG CAT TCA C-3'
	Pre-662-F2	5'-GGC CTT CTG AAG GTC TCC CA-3'
	Pre-662-R2	5'-GCA ACG TGA CTG CGC TGC TG-3'

2.2.6.5 SYBR Green Real Time PCR of pri-miRNA and pre-miRNA

Wild-type allele and variant allele of pri-miRNA or/and pre-miRNA expression level were quantified using Power SYBR® Green PCR master mix (Applied Biosystems). Before quantifying the expression level of pri-miRNA and pre-miRNA, primer specificity and efficiency were checked by performing a serial dilution followed by a standard curve generated by real time PCR machine, ABI 7500 PCR system, (Applied Biosystems). Efficiency of gene-specific primers was calculated using the formula: E = 10 -1/slope - 126, where slope refers to the slope of the standard curve. Gene-specific primers that showed a single peak of dissociation curve were used to perform quantitative of real time PCR.

The amplification reactions were performed in a 12.5µl reaction that contained 1X of Power SYBR Green PCR Master Mix (2X) and 50nM of forward and reverse primers (F1 and R1 for pri-miRNA; F2 and R2 for pre-miRNA). The template used was cDNA (60ng/µl) transcribed from HEK 293T cells. ABI 7500 PCR system, 9600 emulation mode, was used to preform SYBR Green Real Time PCR with the conditions of 1 cycle of UNG incubation at 50°C for 2 minutes, polymerase activation at 95°C for 10 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing or extension at 60°C for 1 minutes. The thermal denaturation step was run to determine the number of products that were present in the reaction. All reactions were run in triplicates, including no template control and internal control. GAPDH and beta-actin genes were the two internal controls used in SYBR Green real time PCR. Three technical repeats were performed to confirm the result consistency. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined for each gene. The relative amount of each pri-miRNA or pre-miRNA to internal controls was

obtained by using the equation $2^{-\Delta\Delta CT}$ method. The relative gene expression was transformed to Log_{10} in order to simplify the presentation of data.

2.2.6.6 TaqMan miRNA Real Time PCR of mature miRNA

Real Time quantitative PCR was performed according to the standard protocols of ABI 7500 system, standard mode (Applied Biosystems). TaqMan® microRNA assays (Applied Biosystems) was used to quantify mature miRNA expression level. Each individual RT reaction has a unique set of PCR primers and TaqMan probe designed for it. Custom made TaqMan Gene Expression Assay was designed for the mature miRNA variant, since the probe is specific. A volume of 12.5µl final reaction was prepared by adding 1 X TaqMan® Fast Advanced Master Mix (2X) and 1 X of TaqMan or Custom TaqMan Gene Expression Assay (20X). Approximately 200 ng/µl of single stranded cDNA of mature miRNA was used. The thermal-cycling profile was performed at 1 cycle of UNG incubation at 50°C for 2 minutes, polymerase activation at 95°C for 20 seconds and 40 cycles of denaturation at 95°C for 3 seconds and annealing or extension at 60°C for 32 seconds. The real time PCR reactions were performed in triplicates. RNU44 and miRNA-16 were used as the internal control in this TaqMan miRNA real time PCR. Three technical repeats were performed. The relative amount of each mature miRNA to internal controls was obtained by using the equation $2^{-\Delta\Delta\ CT}$ method. The relative gene expression was transformed to Log_{10} in order to simplify the presentation of data. Student's paired two-tailed T-test was used to calculate the significant differences of miRNA expression.

- delta C_T ($\Delta\ C_T$) = C_T miRNA - C_T internal control or endogenous control
- delta delta C_T ($\Delta\Delta\ C_T$) = $\Delta\ C_T$ miRNA - $\Delta\ C_T$ empty vector
- Relative Quantification = $2^{-\Delta\Delta\ CT}$

CHAPTER 3 RESULTS

3.1 BLAT search for single nucleotide variation within seed region sequence of mature miRNAs

From the BLAT search of 1048 pre-miRNAs and their 1344 mature miRNAs sequences, a total of 453 single nucleotide variants were found in 299 pre-miRNAs and 151 single nucleotide variant were found in 127 mature miRNAs of *Homo sapiens*. From these 151 **s**ingle nucleotide variant on mature miRNAs, 42 were found on the seed region of 36 mature miRNAs (miRBase 12.0 - miRBase14.0), as shown in Table 3.1. This is because few sets of single nucleotide variant matched to the same mature miRNA sequence. Among these 42 single nucleotide variant, 12 single nucleotide variant on seed region of mature miRNAs (miRBase 16.0) were not available during the selection of candidation miRNAs for functional study. They are miR-3117, miR-3118-2, miR-3118-3, miR-3124, miR-3128, miR-3161, miR-3618, miR-3689b, miR-3910-2, miR-3939, miR-4257 and miR-4293.

Table 3.1: Potential single nucleotide variant within seed region sequence of mature miRNAs by homology search (BLAT result)

miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
hsa-mir-122 MI0000442	5’-TGGAGTGTGACAATGGTGTTTG-3’ 5’-AA**C**GCCATTATCACACTAAATA-3’ (*)	rs41292412	chr18:56118358-56118358	+	C/T
hsa-mir-124-3 MI0000445	5’-CGTGTTCACAGCGGACCTTGAT-3’ 5’-TAA**G**GCACGCGGTGAATGCC-3’ (*)	rs34059726	chr20:61809907-61809907	+	G/T
hsa-mir-125a MI0000469	5’-TCCCTGA**G**ACCCTTTAACCTGTGA-3’ 5’-ACAGGTGAGGTTCTTGGGAGCC-3’ (*)	rs12975333	chr19:56888340-56888340	+	G/T
hsa-mir-146a MI0000477	5’-TGAGAACTGAATTCCATGGGTT-3’ 5’-CCT**C**TGAAATTCAGTTCTTCAG-3’ (*)	rs2910164	chr5:159844996-159844996	+	C/G
hsa-mir-221 MI0000298	5’-ACCT**G**GCATACAATGTAGATTT-3’ 5’-AGCTACATTGTCTGCTGGGTTT-3’ (*)	rs113054794	chrX:45605666-45605666	+	A/C
hsa-mir-379 MI0000787	5’-TGGTAG**A**CTATGGAACGTAGG-3’ 5’-TATGTAACATGGTCCACTAACT-3’ (*)	rs61991156	chr14:101488414-101488414	+	A/G
hsa-mir-431 MI0001721	5’-TGTCTTGCAGGCCGTCATGCA-3’ 5’-CA**G**GTCGTCTTGCAGGGCTTCT-3’ (*)	rs12884005	chr14:101347408-101347408	+	A/G
hsa-mir-449c MI0003823	5’-TAGGCAGTGTATTGCTAGCGGCTGT-3’ 5’-T**T**GCTAGTTGCACTCCTCTCTGT-3’ (*)	rs35770269	chr5:54468124-54468124	+	A/T
hsa-mir-499 MI0003183	5’-TTAAGACTTGCAGTGATGTTT-3’ 5’-AAC**A**TCACAGCAAGTCTGTGCT-3’ (*)	rs3746444	chr20:33578251-33578251	-	C/T
hsa-mir-513a-1 MI0003191	5’-TTCACA**G**GGAGGTGTCAT-3’ 5’-TAAATTTCACCTTTCTGAGAAGG-3’ (*)	rs35027589	chrX:146295068-146295067	-	-/C
hsa-mir-518d MI0003171	5’-CTCTAGAGGGAAGCACTTTCTG-3’ 5’-CAAAGC**G**CTTCCCTTTGGAGC-3’ (*)	rs73602910	chr19:54238189-54238189	+	A/G
hsa-mir-518e MI0003169	5’-CT**C**TAGAGGGAAGCGCTTTCTG-3’ 5’-AAAGCGCTTCCCTTCAGAG-3’ (*)	rs74177813	chr19:54233109-54233109	+	-/C
	5’-CTCTA**G**AGGGAAGCGCTTTCTG-3’ 5’-AAAGCGCTTCCCTTCAGAG-3’ (*)	rs34416818	chr19:54233113-54233112	+	-/A
	5’-CTCTAG**A**GGGAAGCGCTTTCTG-3’ 5’-AAAGCGCTTCCCTTCAGAG-3’ (*)	rs74177814	chr19:54233114-54233113	+	-/G
hsa-mir-557 MI0003563	5’-GTTTGCA**C**GGGTGGGCCTTGTCT-3’	rs78825966	chr1:168344829-168344829	+	C/T
hsa-mir-585 MI0003592	5’-TGG**G**CGTATCTGTATGCTA-3’	rs62376935	chr5:168690635-168690635	+	C/T

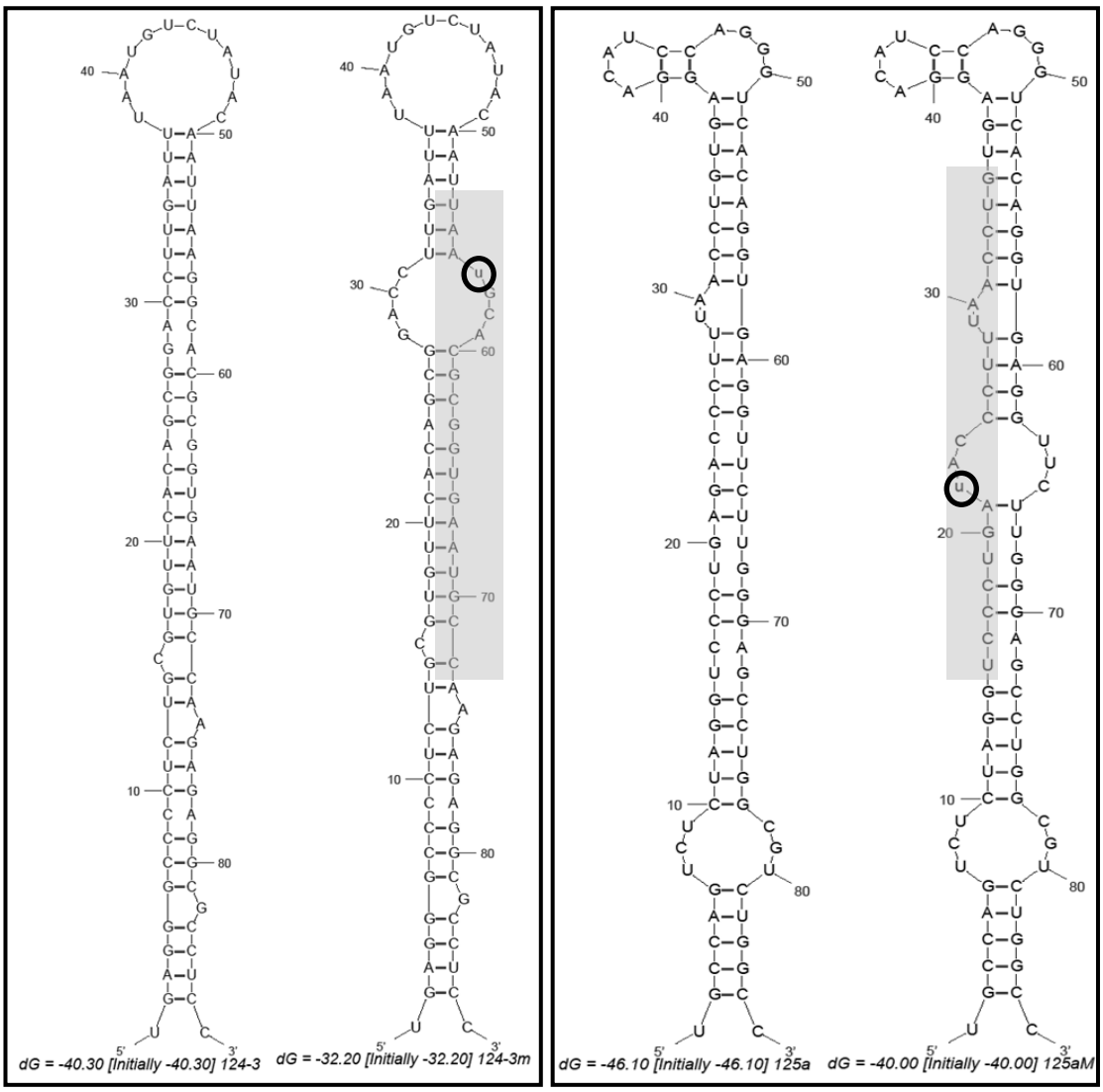
Table 3.1: continued

miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
hsa-mir-605 MI0003618	5’-T**A**AATCCCATGGTGCCTTCTCCT-3’	rs113212828	chr10:53059349-53059349	+	A/G
hsa-mir-627 MI0003641	5’-GTAG**T**GAGTCTCTAAGAAAAGAGGA-3’	rs2620381	chr15:42491848-42491848	+	A/C
hsa-mir-662 MI0003670	5’-TCCCAC**G**TTGTGGCCCAGCAG-3’	rs9745376	chr16:820249-820249	+	A/G
hsa-mir-936 MI0005758	5’-AC**A**GTAGAGGGAGGAATCGCAG-3’	rs79924817	chr10:105807928-105807928	+	C/T
hsa-mir-938 MI0005760	5’-T**G**CCCTTAAAGGTGAACCCAGT-3’	rs12416605	chr10:29891260-29891260	+	C/T
hsa-mir-941-4 MI0005766	5’-CACCC**G**GCTGTGTGCACATGTGC-3’	rs76606291	chr20:62551216-62551216	+	A/G
hsa-mir-1268 MI0006405	5’-CGGGC**G**TGGTGGTGGGGG-3’	rs28599926	chr15:22513271-22513271	+	C/T
hsa-mir-1276 MI0006416	5’-TAAA**G**AGCCCTGTGGAGACA-3’	rs34381260	chr15:86313795-86313794	+	-/T
hsa-mir-1299 MI0006359	5’-TTCT**G**GAATCCTACGTGAGGGA-3’	rs76266132	chr9:69002317-69002317	+	C/T
hsa-mir-1304 MI0006371	5’-TT**T**GAGGCTACAGTGAGATGTG-3’	rs79759099	chr11:93466909-93466909	+	A/G
	5’-T**T**TGAGGCTACAGTGAGATGTG-3’	rs76857625	chr11:93466910-93466910	+	A/G
hsa-mir-3117 MI0014130	5’-ATA**G**GACTCATATAGTGCCAG-3’	rs12402181	chr1:67094171-67094171	+	A/G
hsa-mir-3118-2 MI0014132	5’-TGTGACT**G**CATTATGAAAATTCT-3’	rs11488501	chr1:143424165-143424165	+	C/T
hsa-mir-3118-3 MI0014133	5’-TGTGACT**G**CATTATGAAAATTCT-3’	rs11488501	chr1:143424165-143424165	+	C/T
hsa-mir-3124 MI0014140	5’-TT**C**GCGGGCGAAGGCAAAGTC-3’	rs12081872	chr1:249120584-249120584	+	C/T
hsa-mir-3128 MI0014145	5’-TCTG**G**CAAGTAAAAAACTCTCAT-3’	rs61130794	chr2:178120681-178120681	+	G/T
hsa-mir-3161 MI0014191	5’-CTGATA**A**GAACAGAGGCCCAGAT-3’	rs11382316	chr11:48118350-48118349	+	-/A
	5’-CTGATAA**G**AACAGAGGCCCAGAT-3’	rs35834266	chr11:48118351-48118350	+	-/A
hsa-mir-3618 MI0016008	5’-TGT**C**TACATTAATGAAAAGAGC-3’	rs12159555	chr22:20073323-20073323	+	C/G
hsa-mir-3689b MI0016411	5’-TGTGAT**A**TCATGGTTCCTGGGA-3’	rs116838571	chr9:137742067-137742067	+	A/T
hsa-mir-3910-2 MI0016431	5’-AAAAGG**C**ATAAAACCAAGACA-3’	rs76084273	chr9:94398600-94398600	+	A/C
hsa-mir-3939 MI0016596	5’-TACGCG**C**AGACCACAGGATGTC-3’	rs76608449	chr6:167411333-167411333	+	C/G
	5’-TACGC**G**CAGACCACAGGATGTC-3’	rs75823810	chr6:167411334-167411334	+	C/T
	5’-TACGC**G**CAGACCACAGGATGTC-3’	rs73024232	chr6:167411337-167411337	+	A/G
hsa-mir-4257 MI0015856	5’-CCAGA**G**GTGGGGACTGAG-3’	rs74743733	chr1:150524468-150524468	+	A/G
hsa-mir-4293 MI0015826	5’-CAGC**C**TGACAGGAACAG-3’	rs12220909	chr10:14425221-14425221	+	C/G

Asterisk (*) shows the mature miRNA sequence from the complementary miRNA that is functional.
The red coloured, bold and underlined nucleotide indicates the variant allele. Blue highlighted columns show the potential single nucleotide variant within seed region sequence of mature miRNAs by homology search after secondary structural prediction (MFOLD).

3.2 Prediction of secondary RNA structures for the wild-type allele and variant allele of pre-miRNAs

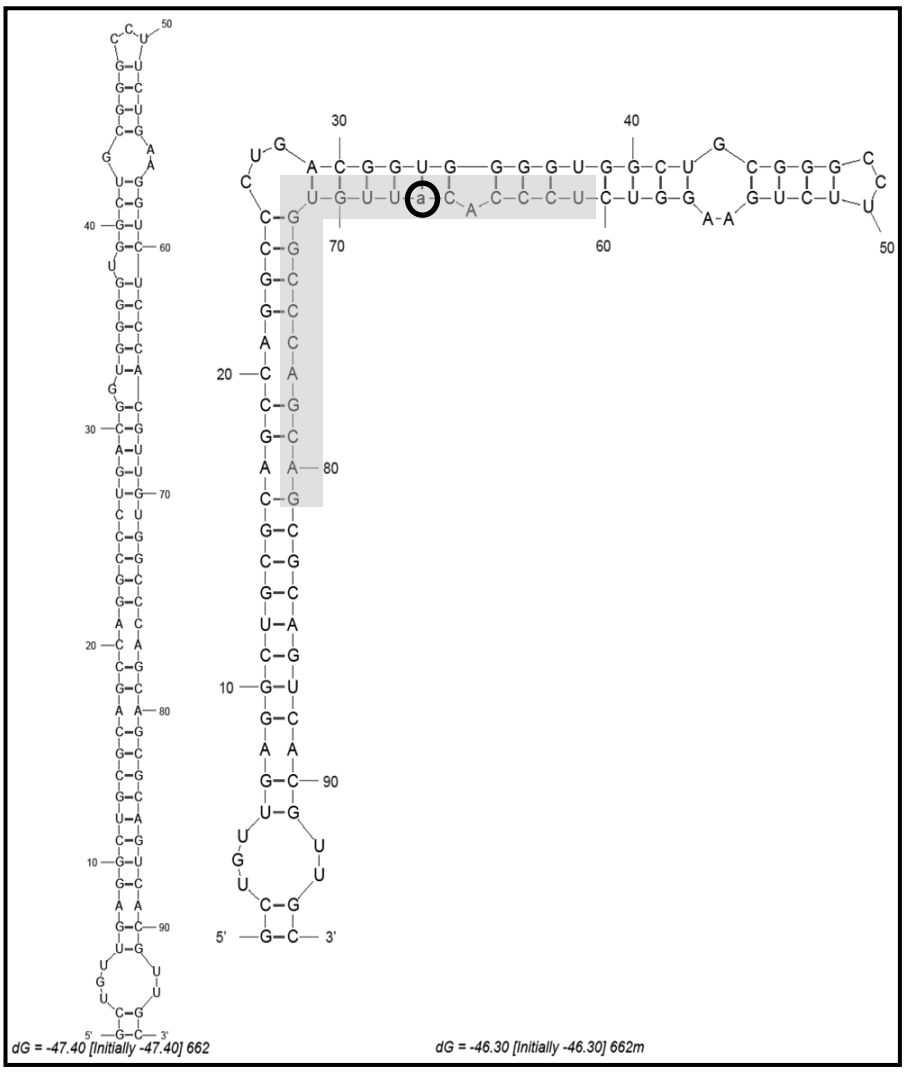
Secondary stem loop structures of these 42 single nucleotide variant that were found within the seed region sequences of 5'-end mature miRNAs was predicted by using MFOLD web server. After individual and careful checking of the secondary structures for 42 pairs of wild-type and variant allele , only 14 sets of single nucleotide variant within seed region sequence of mature miRNAs were found to show structural difference and minimal folding free energy deviation, as shown in Figure 3-1. Among these single nucleotide variant within seed region sequence of mature miRNAs, miR-125a, miR-124-3 and miR-662 were randomly chosen as the study subjects.



miR-124-3 miR-124-3 mutant miR-125a miR-125a mutant

SNP ID: rs34059726 SNP ID: rs12975333

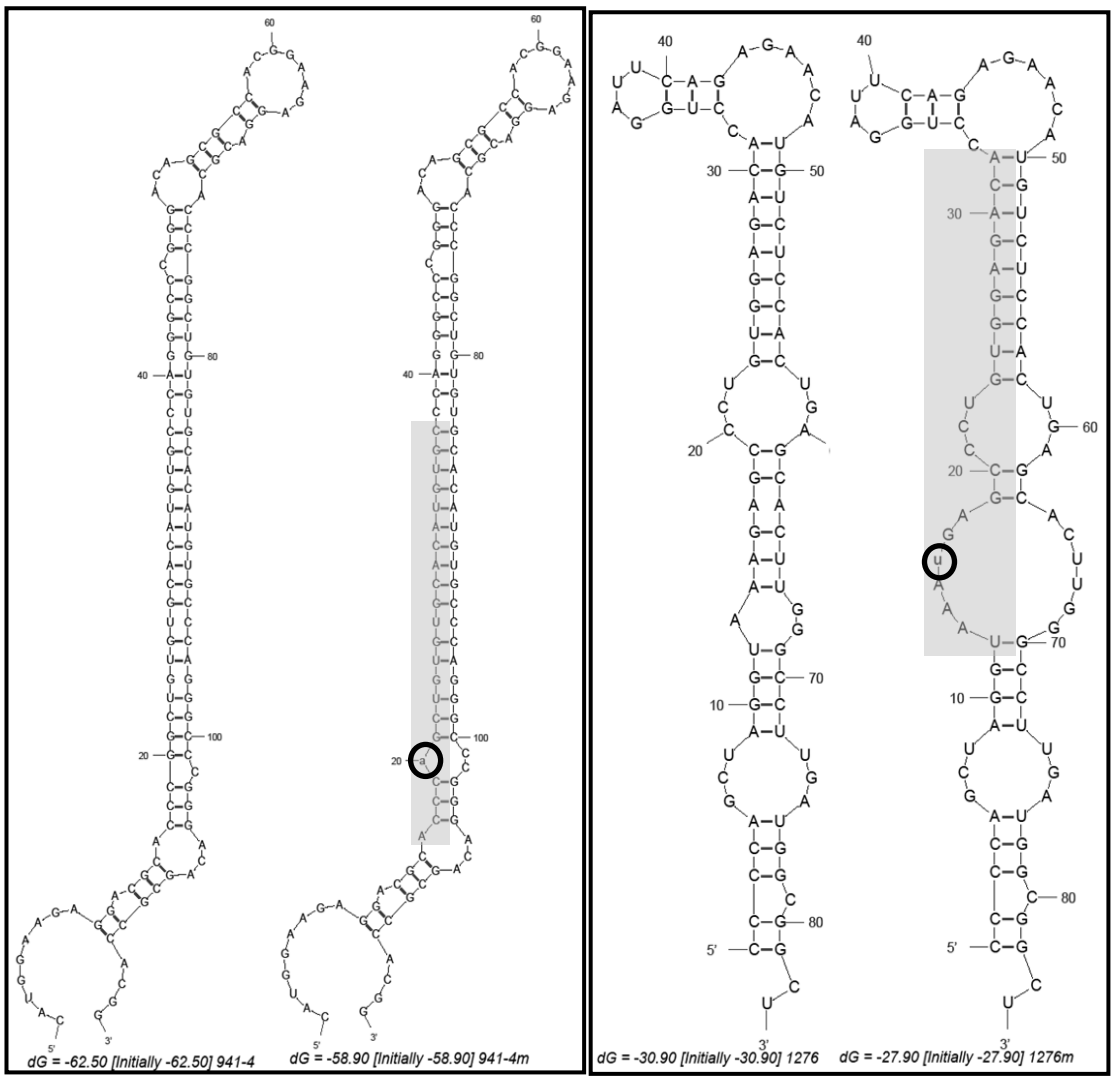
Figure 3-1: Secondary structures of the wild type allele and variant allele pre-miRNAs as predicted by MFOLD.
The mature miRNA sequences were shadowed and the black circle indicates the variant allele. Minimal folding free energies (dG) calculated by MFOLD were indicated.



miR-662 miR-662 mutant

SNP ID: rs9745376

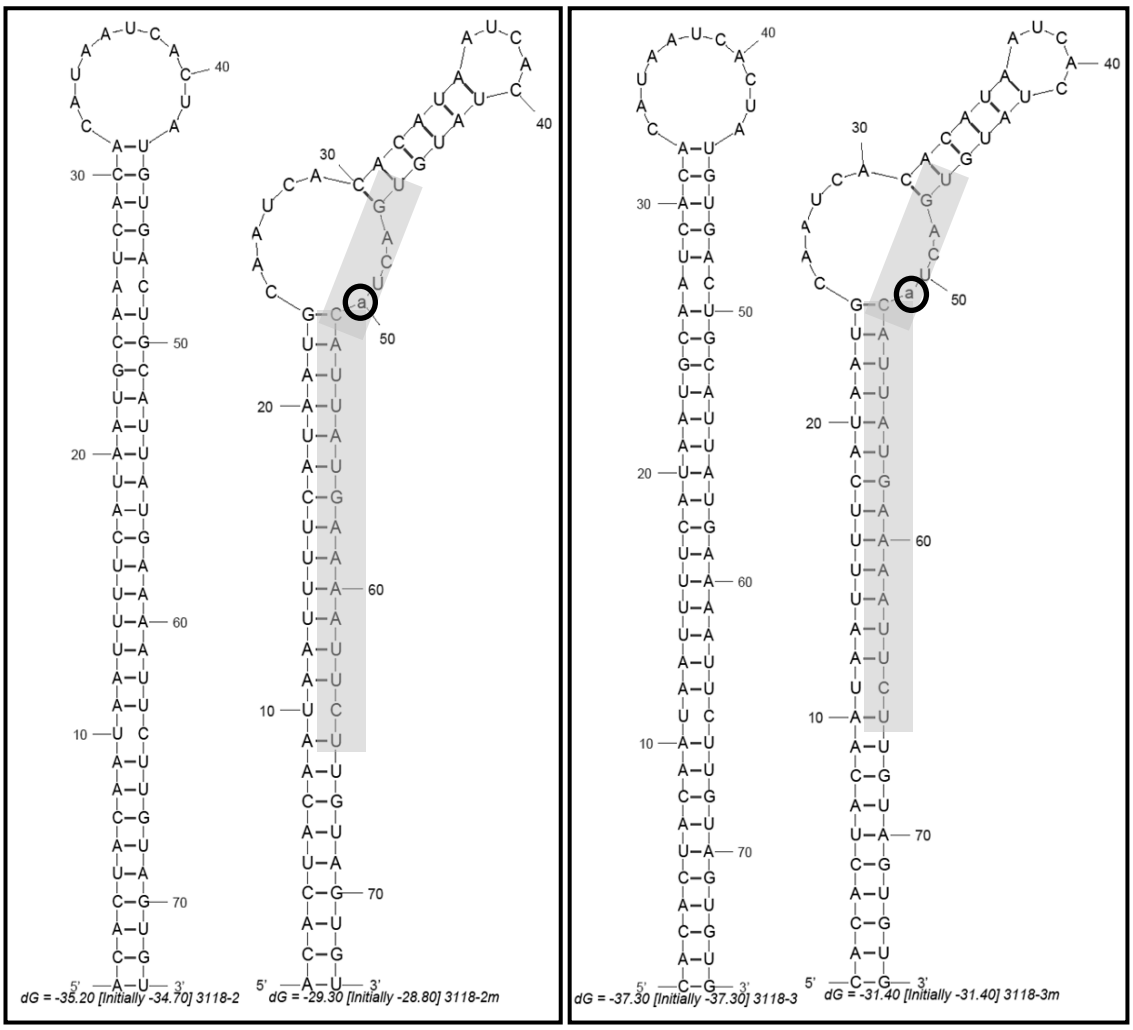
Figure 3.1 (continued)



miR-941-4 miR941-4 mutant miR-1276 miR1276 mutant

SNP ID: rs76606291 SNP ID: rs34381260

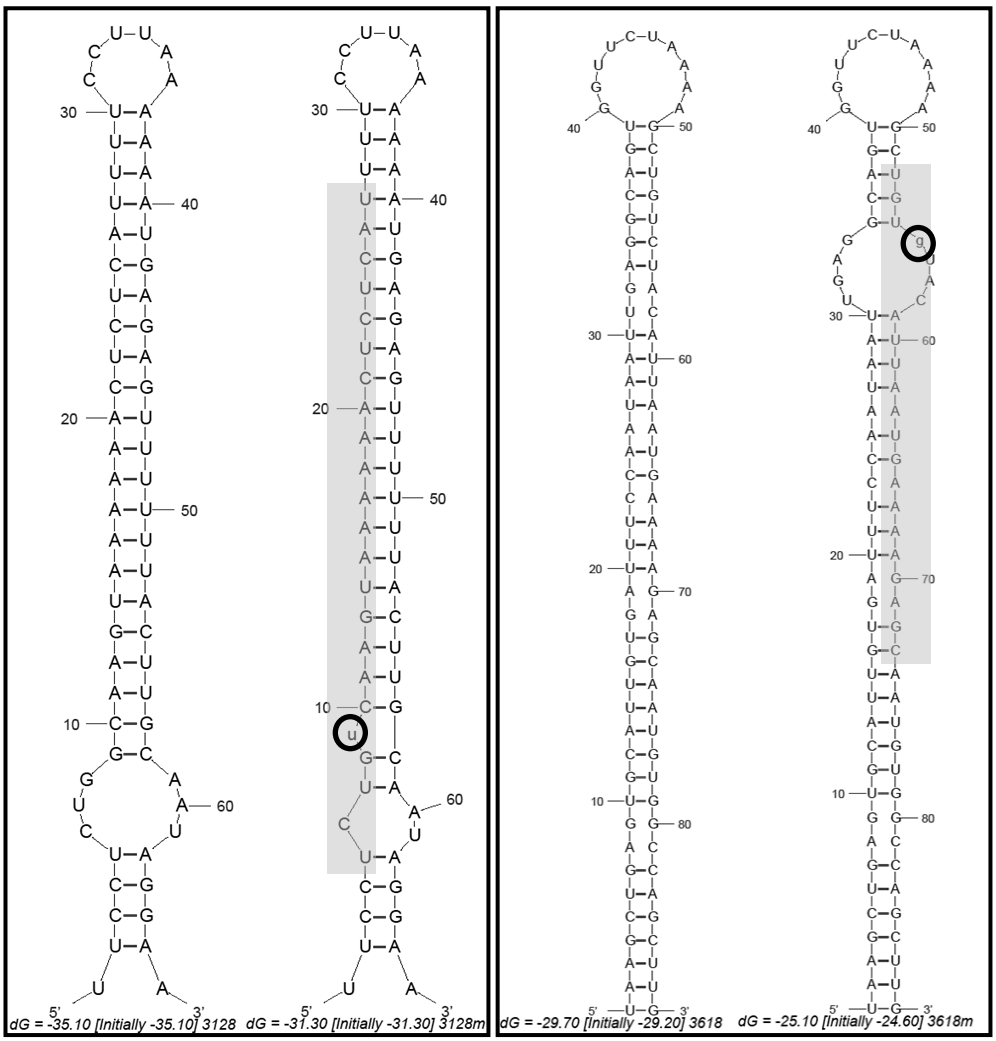
Figure 3.1 (continued)



miR-3118-2 miR-3118-2 mutant SNP ID: rs11488501

miR-3118-3 miR-3118-3 mutant SNP ID: rs11488501

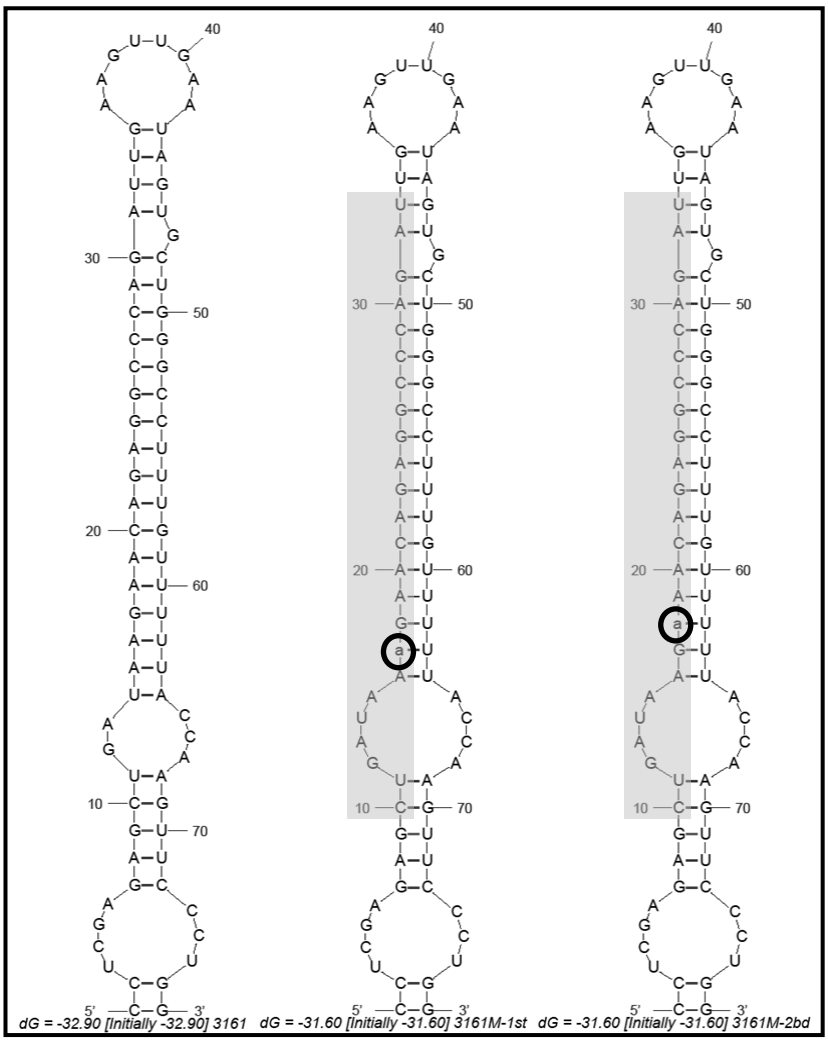
Figure 3.1 (continued)



miR-3128 miR-3128 mutant miR-3618 miR-3618 mutant

SNP ID: rs11382316 SNP ID: rs12159555

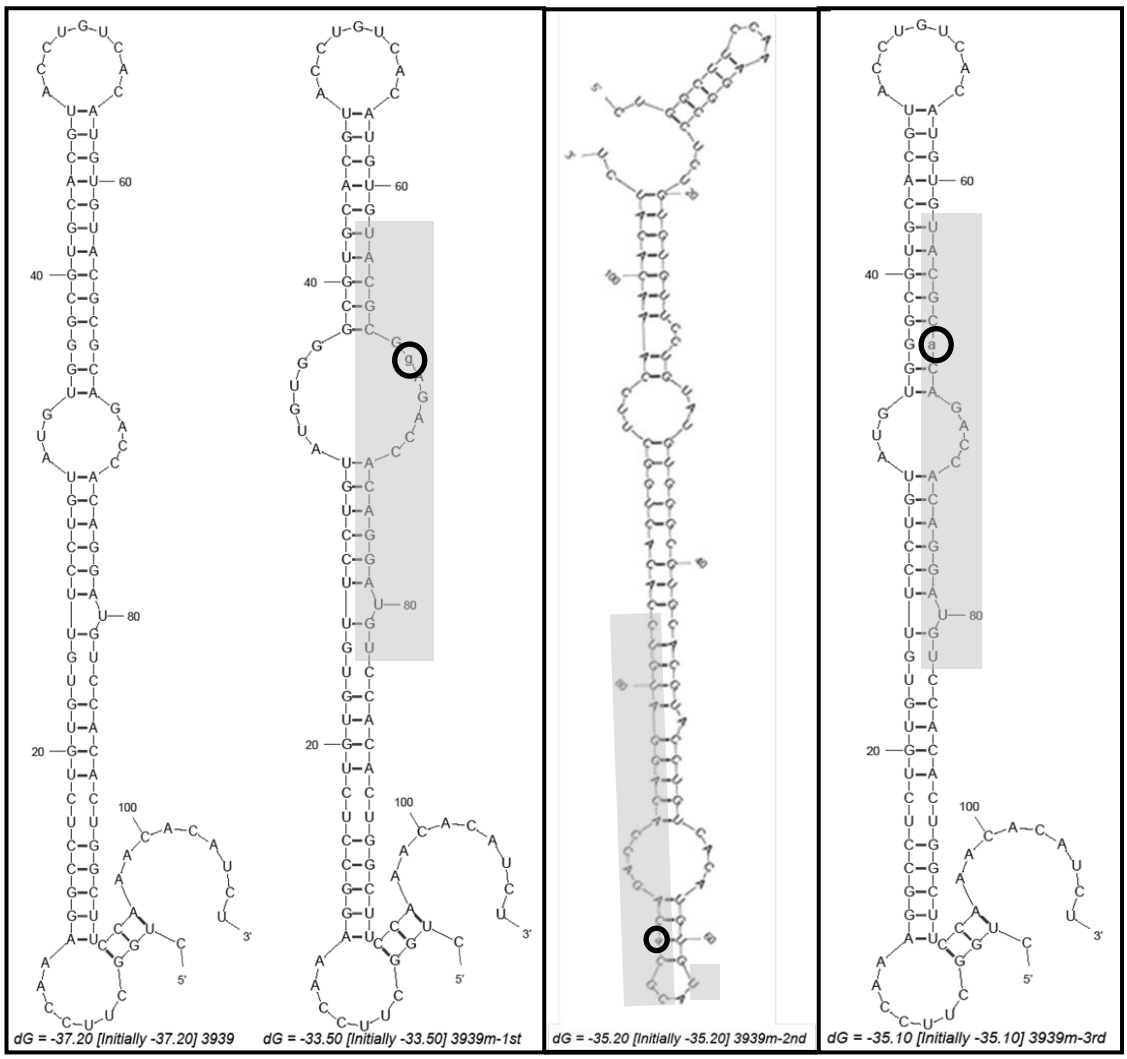
Figure 3.1 (continued)



miR-3161 miR-3161 mutant 1 miR-3161 mutant 2

SNP ID: rs61130794 SNP ID: rs35834266

Figure 3.1 (continued)



miR-3939 miR-3939 mutant 1 miR-3939 mutant 2 miR-3939 mutant 3

SNP ID: rs76608449 SNP ID: rs75823810 SNP ID: rs73024232

Figure 3.1 (continued)

3.3 Genomic DNA extracted from control human blood

Human genomic DNA was successfully isolated from blood samples with the conventional phenol-chloroform extraction method (as mentioned in section 2.2.4.1). The absorbance ratio of 260nm over 280nm (A_{260}/A_{280}) from spectrophotometer was 1.74, which indicate that the extracted DNA was relatively pure. The quality of DNA sample was determined by electrophoresis (Figure 3-2). DNA was run on a 0.8% agarose gel and visible bands were observed in the well corresponding to the high molecular weight of genomic DNA which is greater than 23 kilobase. No degradation of the genomic DNA was observed.

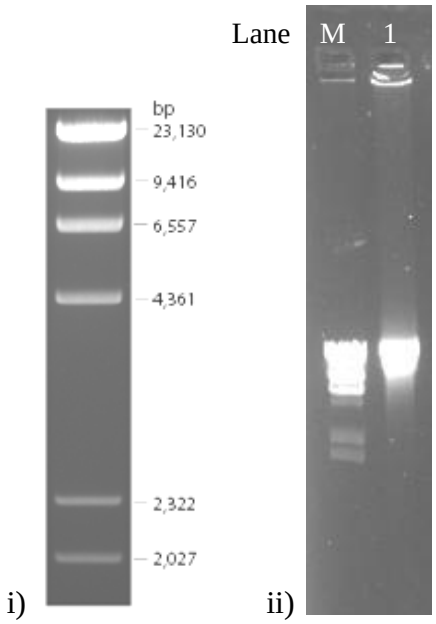


Figure 3-2: Genomic DNA from human blood.
(i) NEB Lambda HindIII ladder (ii) Agarose gel of extracted human genomic DNA. Lane M indicates the Lambda HindIII ladder and lane 1 indicates genomic DNA with the size greater than 23kb.

3.4 Polymerase Chain Reaction of wild type allele of pri- microRNAs

In order to generate the wild-type allele of the pri-miRNAs studied, the wild-type alleles (miR-125a, miR-124-3 and miR-662) were amplified from normal human genomic DNA by PCR. The PCR product was further purified using Gel extraction kit. Purified wild type allele of pri-miRNA bands were run on 1.5% agarose gel and visible bands were observed (Figure 3-3). Only one significant band with expected fragment size (between 400bp and 500bp) was seen from each PCR reaction, showing that the primers were specific enough to amplify wild-type pri-miRNA.

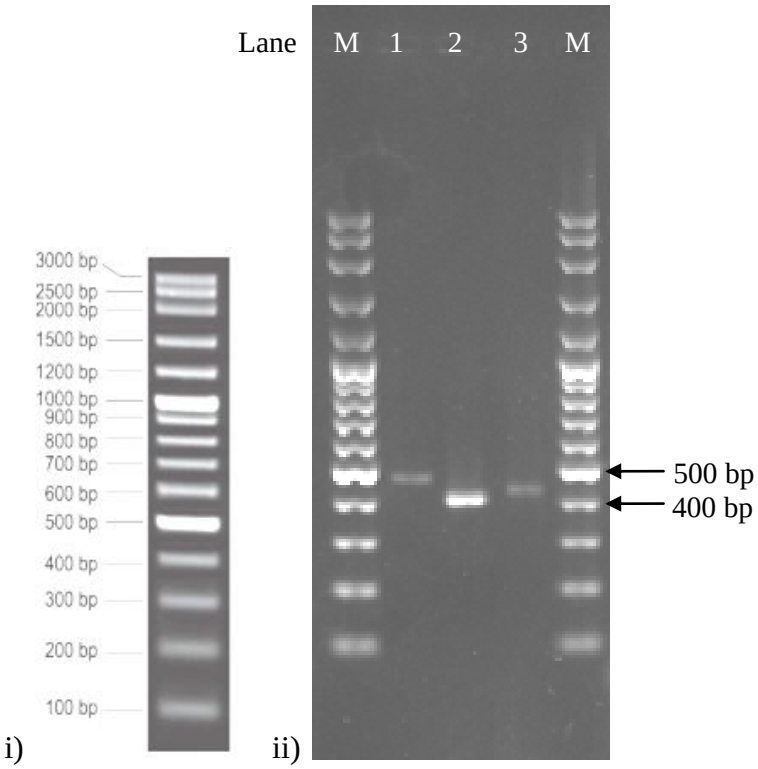


Figure 3-3: PCR of wild type pri-miRNA fragment.
(i) Vivantis 100 bp plus DNA ladder (ii) Purified PCR products of pri-miRNA with specific primers. Lane M is the 100 bp plus ladder lane 1 is pri-miR-125a (459 bp), lane 2 is pri-miR-124-3 (399 bp) and lane 3 is pri-miR-662 (424 bp).

3.5 Cloning and transformation of pri-miRNAs

Purified PCR products of wild-type pri-miRNA were successfully cloned into PCR 4-TOPO vector or pGEM®-T Easy Vector. Colonies were picked for colony PCR (Figure 3-4) to select for positive transformant that contained the purified DNA fragment. The primers used in colony PCR are specific forward primer and reverse primer of vector to amplify the cloned DNA fragment. The bands right below the wells were the bacteria genomic DNA. Negative controls (without bacteria colony sample) were included. No band was observed in the negative controls showing no contamination.

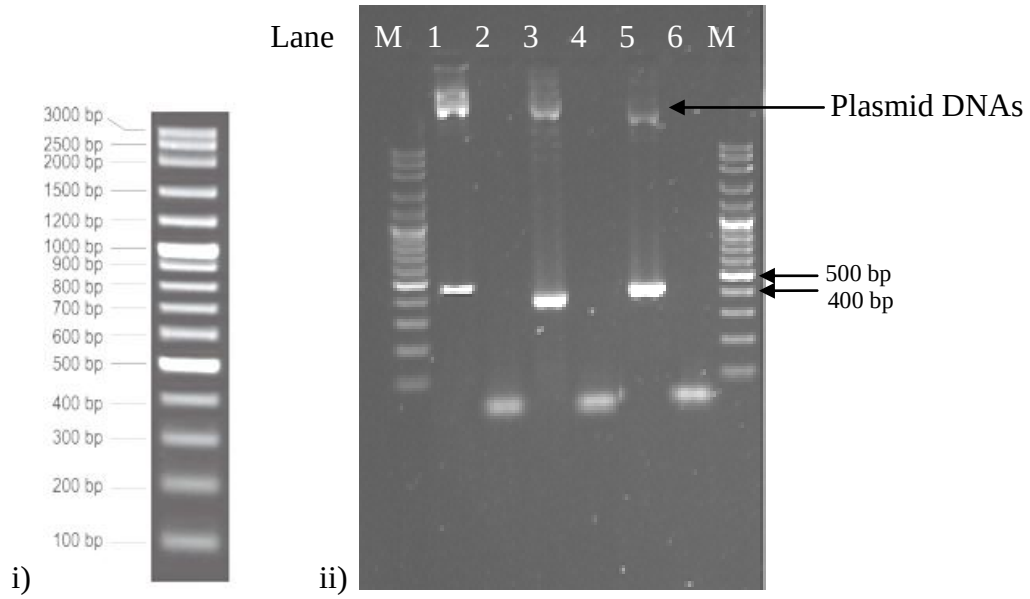


Figure 3-4: Colony PCR of pri-miRNA fragment from bacteria colony:
(i) Vivantis 100 bp plus DNA ladder (ii) Colony PCR of pri-miRNAs. Lane M is the 100 bp plus ladder lane 1 is pri-miR-125a (459 bp), lane 3 is pri-miR-124-3 (399 bp) and lane 5 is pri-miR-662 (424 bp). Lanes 2, 4 and 6 are negative controls.

3.6 Plasmids of TA vector

Positive transformant were successfully cultured in Luria-Bertani broth overnight. Plasmids were extracted by using DNA plasmid extraction kit and were run in 0.8% agarose gel to visualize the product of extraction (Figure 3-5). The estimated size of supercoiled plasmids and their insertions are 5.8 kb for miR-125a gene in pcDNA™ 3.3-TOPO® TA, 4 kb for miR-124-3 gene in PCR 4-TOPO vector and 3 kb for miR-662 gene in pGEM®-T Easy Vector.

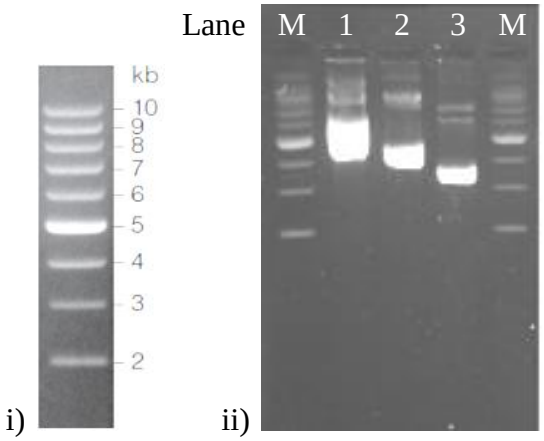


Figure 3-5: Plasmids containing wild type pri-miRNA:
(i) Promega supercoiled DNA ladder (ii) Plasmids of positive clone on 0.8 % agarose gel. Lane M is the supercoiled DNA ladder, lane 1 is pcDNA – pri-miR-125a (5.8 Kb), lane 2 is PCR 4 TOPO – pri-miR-124-3 (4.35 Kb) and lane 3 is pGEM®-T – pri-miR-662 (3.4 Kb).

PCR was performed on the extracted recombinant plasmids (plasmid pri-miR-125a, plasmid pri-miR124-3 and plasmid pri-miR-662) and the PCR products were observed through 1.5% agarose gel electrophoresis (Figure 3-6). This was to confirm successful cloning of wild-type gene of pri-miRNA fragment into the extracted plasmids. The PCR product showing molecular weight greater than 3kb was the plasmid DNAs. Only primer dimers were observed in the negative control proving no DNA contamination in the PCR. Positive transformant were later subjected to sequencing (Figure 3-15 to Figure 3-17).

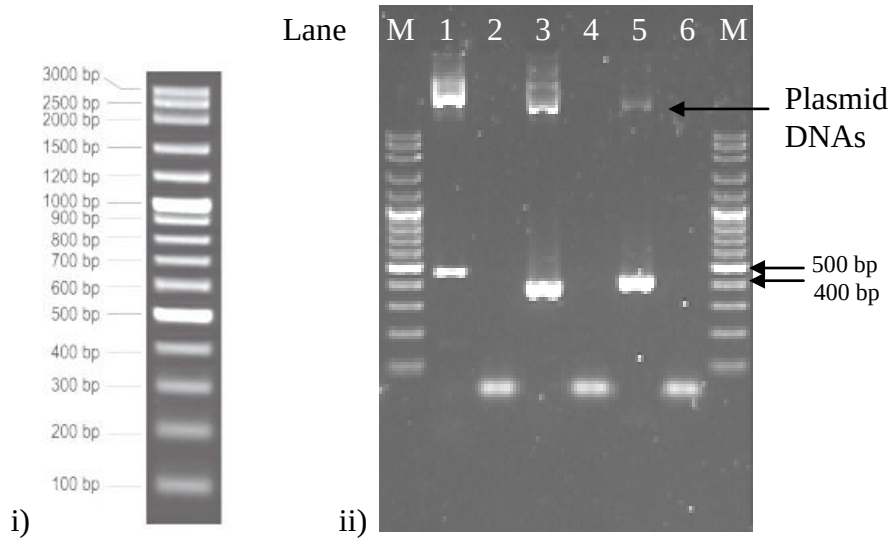


Figure 3-6: PCR of plasmid pri-miRNA:
(i) Vivantis 100bp plus DNA ladder (ii) PCR of positive transformant on 1.5% agarose gel. Lane M is the 100bp plus ladder, lane 1 is pri-miR-125a (459 bp), lane 3 is pri-miR-124-3 (399 bp) and lane 5 is pri-miR-662 (424 bp). Lanes 2, 4 and 6 are negative controls.

3.7 Subcloning of pri-miRNAs sequencing vectors to pcDNA ™ 3.1/Zeo (-) vector

Positive transformant containing the correct wild-type allele pri-miRNA fragment were sub-cloned into mammalian expression vector, pcDNA ™ 3.1/Zeo (-) vector. Before sub-cloning, pcDNA ™ 3.1/Zeo (-) vector and the extracted plasmids were successfully cleaved with restriction enzymes to obtain a linearlized pcDNA™ vector (5kb) and the fragment of wild-type gene cleared from vector (Figure 3-7). The digested fragments were visualized using agarose gel electrophoresis. Bands between 400bp and 500bp were the fragment of wile-type gene.

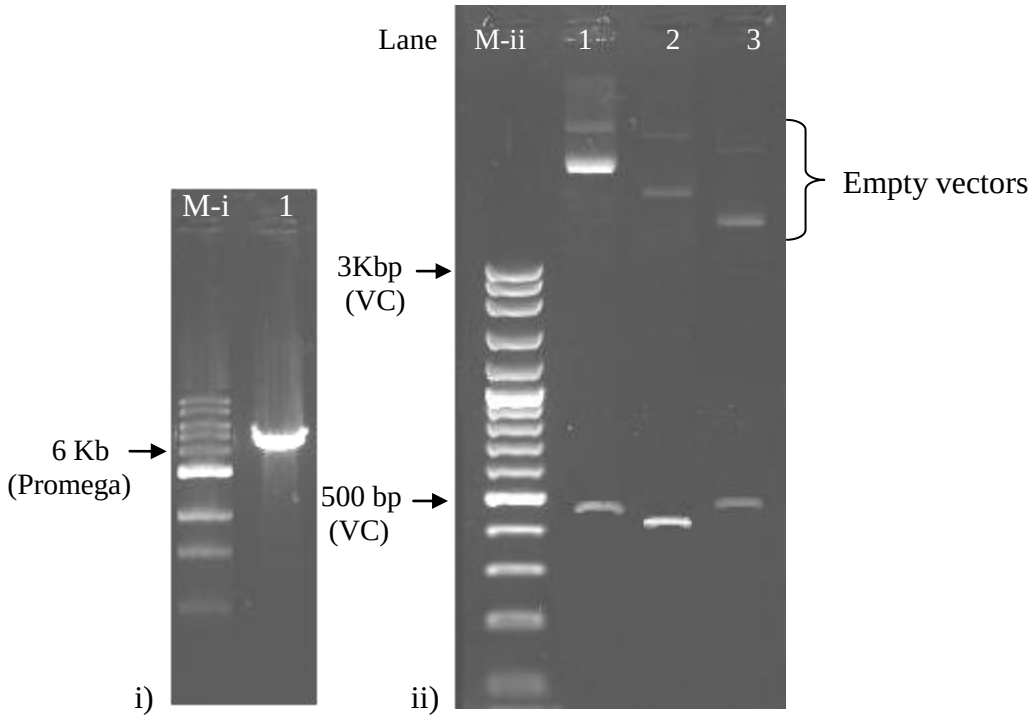


Figure 3-7: Digested expression vector and wild type pri-miRNA fragment:
(i) Linearlized pcDNATM 3.1 vector by restriction enzyme. Lane M-i is the supercoiled DNA ladder-and lane 1 is the linearlized pcDNA vector. (ii) Fragment of pri-miRNA genes to be subcloned into pcDNA TM 3.1 vector. Lane M-ii is the 100bp plus DNA ladder, lane 1 is pri-miR-125a (459 bp), lane 2 is pri-miR-124-3 (399 bp) and lane 3 is pri-miR-662 (424 bp).

Colony PCR was used to check the successful subcloning of wild-type allele pri-miRNA fragment into the linearlized pcDNA^{TM} 3.1 vectors (Figure 3-8). Few colonies were picked for PCR to increase the chances of obtaining positive transformant. The primers used are specific forward and reverse primers of pcDNA^{TM} vector to amplify the cloned wild-type gene of pri-miRNA fragment, since orientation of the insertion is important. No amplification was observed in the negative controls.

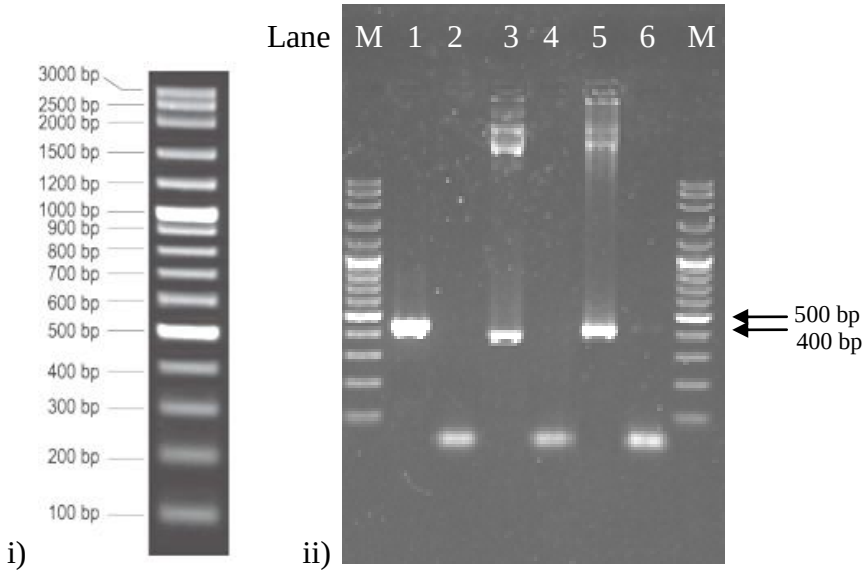


Figure 3-8: PCR of pri-miRNA fragment from expression vector:
(i) Vivantis 100 bp plus DNA ladder (ii) Colony PCR for pri-miRNAs that successfully subcloned into pcDNATM 3.1 vector. Lane M is 100 bp plus DNA ladder, lane 1 is pcDNA pri-miR-125a, lane 2 is pcDNA pri-miR-124-3 and lane 3 is pcDNA pri-miR-662. Lanes 2, 4, 6 and 8 are negative controls with distilled water as template.

Positive clones identified through colony PCR were cultured in LB broth to obtain the pcDNA^{TM} 3.1 vectors. Plasmids were successfully extracted and were run in 0.8% agarose gel (Figure 3-9). The visible bands showed the expected molecular weight, corresponding to approximately 5 kb for all two supercoiled plasmids.

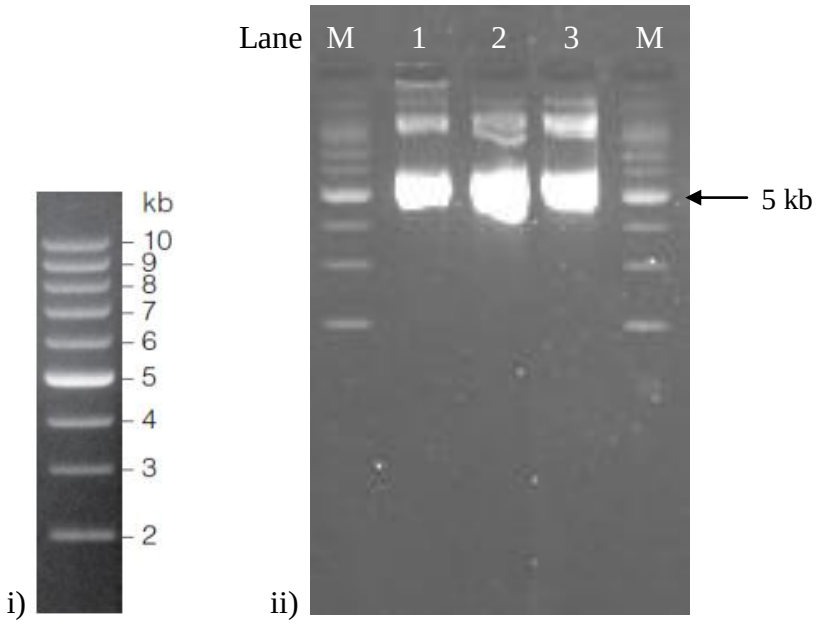


Figure 3-9: Plasmid containing wild type pri-miRNA:
(i) Promega supercoiled DNA ladder (ii) Plasmids of positive clone of pri-miRNAs in pcDNATM 3.1 vector. Lane M is supercoiled DNA ladder, lane 1 is pcDNA pri-miR-125a, lane 2 is pcDNA pri-miR-124-3 and lane 3 is pcDNA pri-miR-662.

PCR was performed by using the extracted pcDNA plasmids as template. This is to confirm the succession of wild-type allele of pri-miRNA fragment inserted in the extracted plasmids before subjected for sequencing. Electrophoresis (1.5% of agarose gel) was run to check the products (Figure 3-10). Only primer dimers were observed in the negative control proving no DNA contamination in the PCR. Positive transformant were later subjected to sequencing (Figure 3.14, 3.15 and 3.16).

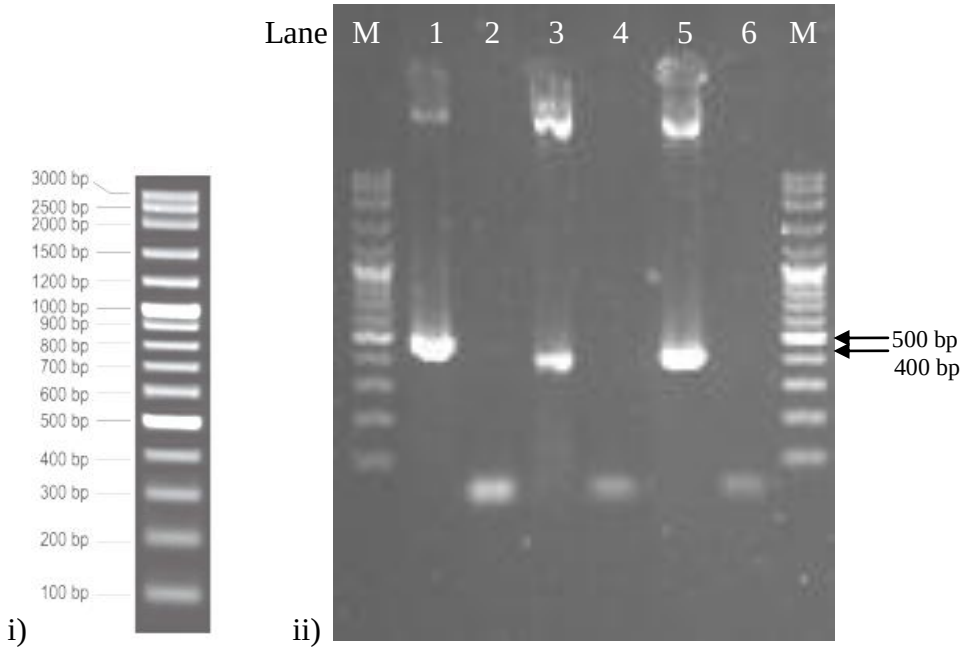


Figure 3-10: PCR of positive clone of expression vector containing wild type pri-miRNA:
(i) Vivantis 100 bp plus DNA ladder (ii) PCR for positive clone of wild-type allele pri-miRNAs in pcDNATM 3.1 vector. Lane M is supercoiled DNA ladder, lane 1 is pcDNA pri-miR-125a, lane 3 is pcDNA pri-miR-124-3 and lane 5 is pcDNA pri-miR-662. Lanes 2, 4, 6 and 8 are negative controls with water as template.

3.8 Mutagenesis of pcDNA pri-miRNAs

Site-directed mutagenesis of the wild-type allele of pri-miRNA fragment in pcDNA^{TM} 3.1 vectors was successfully performed through conventional PCR methods (as mentioned in 2.2.4.11). The colonies obtained from mutagenesis were checked through colony PCR for selection of positive transformant. The amplified products were visualized through 1.5% agarose gel electrophoresis (Figure 3-11). The primers used were specific forward and reverse primer of pcDNA TM 3.1/Zeo (-) vector to amplify and check orientation of the cloned mutagenized pri-miRNA fragment.

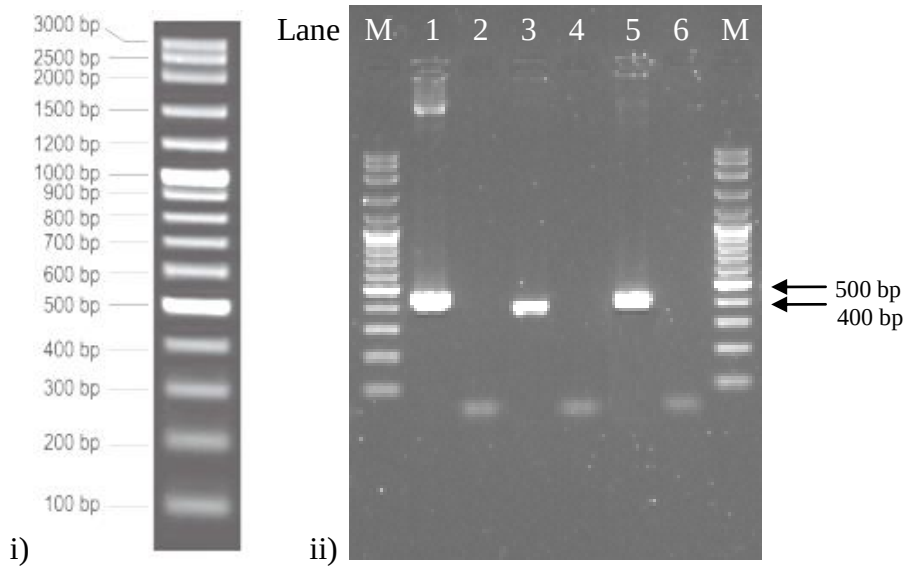


Figure 3-11: PCR of mutagenized pri-miRNA fragment.
(i) Vivantis 100 bp plus DNA ladder (ii) Colony PCR for pri-miRNAs that treated with site-directed mutagenesis. Lane M is the 100 bp plus DNA ladder, lane 1 is pcDNA pri-miR-125aM, lane 3 is pcDNA pri-miR-124-3M, lane 5 is pcDNA pri-miR-662M. Lane 2, 4, 6 and 8 are negative controls.

Colonies harbouring mutagenized pri-miRNA fragment were cultured in LB broth. All two supercoiled recombinant plasmids showed a molecular weight of 5kb, which corresponds to the total size of the mutagenized gene of pri-miRNA and the pcDNA vector (Figure 3-12).

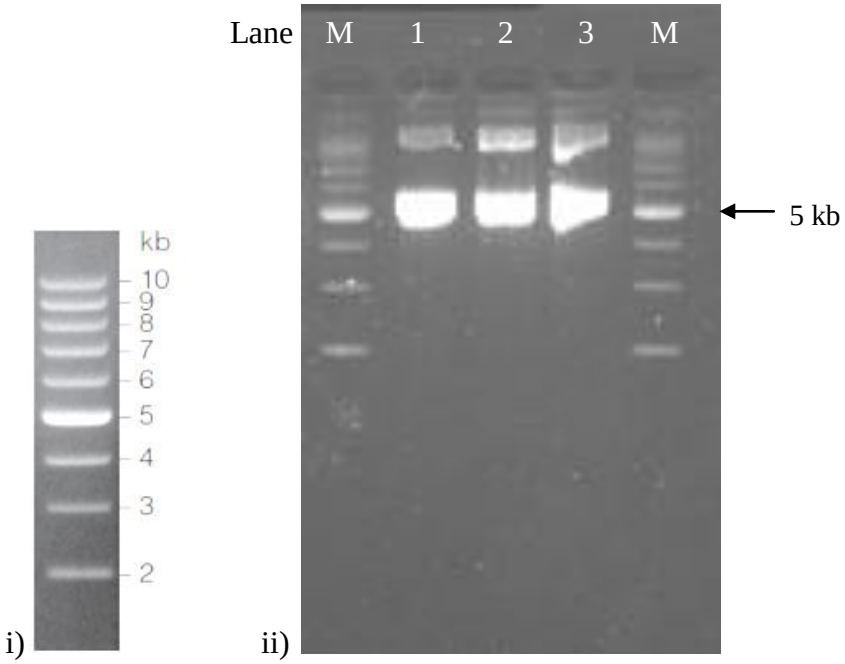


Figure 3-12: Positive transformant containing mutagenized pri-miRNA:
(i) Promega supercoiled DNA ladder (ii) Plasmids of positive transformant for mutagenized pri-miRNAs. Lane M is the supercoiled DNA ladder, lane 1 is pcDNA pri-miR-125aM, lane 2 is pcDNA pri-miR-124-3M and lane 3 is pcDNA pri-miR-662M.

Introduction of the mutagenized allele to pcDNA plasmids were amplified and the PCR products were visualized using 1.5% agarose gel electrophoresis (Figure 3-13). Primers used are specific forward primer and reverse primer of vector. Plasmids harbouring the insertions of wild type and mutagenized gene of pri-miRNAs were sent for sequencing.

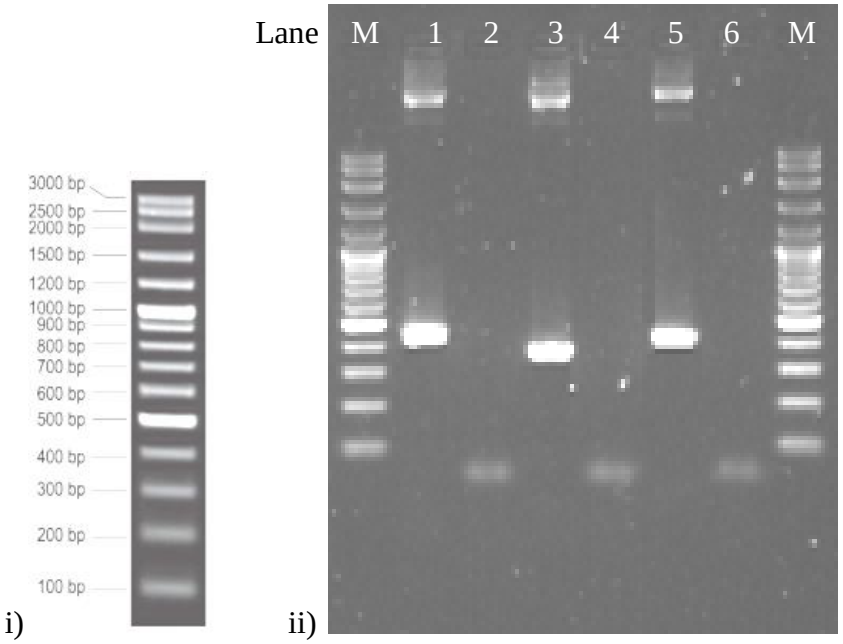


Figure 3-13: PCR of mutagenized pri-miRNA fragment:
(i) Vivantis 100 bp plus DNA ladder (ii) PCR of positive plasmids for mutagenized pri-miRNAs. Lane M is the 100 bp DNA ladder, 1 is pcDNA pri-miR-125aM, lane 2 is pcDNA pri-miR-124-3M and lane 3 is pcDNA pri-miR-662M. Lane 2, 4, 6 and 8 are negative controls.

3.9 DNA sequencing and sequence alignment of pri-miRNAs from database, pcDNA pri-miRNAs and mutagenized pcDNA pri-miRNAs.

DNA sequence from both recombinant plasmids that contained wild-type pri-miRNA gene and mutagenized pri-miRNA gene were used to align with the sequence of pri-miRNA extracted from miRBase database. Sequencing was performed to confirm the sequence of wild type pri-miRNA gene and mutagenized pri-miRNA were individually introduced into the mammalian expression vector, pcDNA^{TM} 3.1 vector. The sequence alignment showed that single nucleotide mutation was successfully introduced into the seed region of mature miRNA of all two wild-type of pri-miRNA (pri-miR-125a, pri-miR-124-3 and pri-miR-662). The mutagenized allele was designed based on the chosen SNP allele that was found to have homology match to the seed region of 5'-end mature miRNA (as shown in Table 3.1). In Figure 3-14, Figure 3-15 and Figure 3-16, the dashed box indicates the mature miRNA sequence and the circled area was the site where mutagenesis took place.

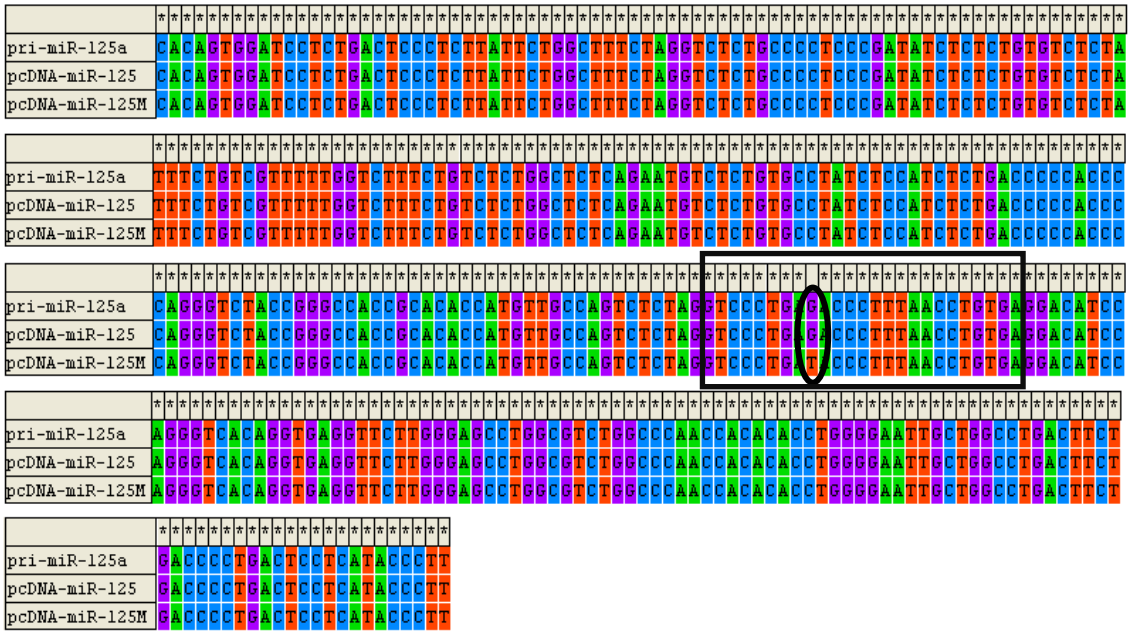


Figure 3-14: Sequence alignment of pri-miR-125a from miRBase database, recombinant plasmid of pri-miR-125a (pcDNA-miR-125a) and recombinant plasmid of mutagenized pri-miR-125a (pcDNA-miR-125aM).

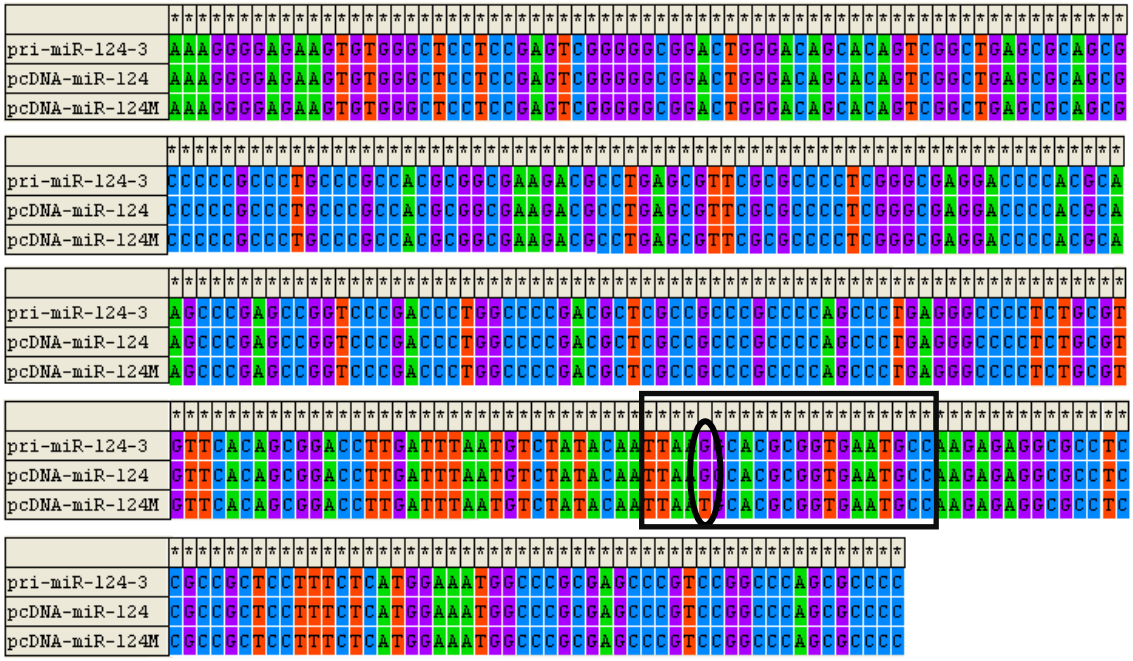


Figure 3-15: Sequence alignment of pri-miR-124-3 from miRBase database, recombinant plasmid of pri-miR-124-3 (pcDNA-miR-124) and recombinant plasmid of mutagenized pri-miR-124-3 (pcDNA-miR-124M).

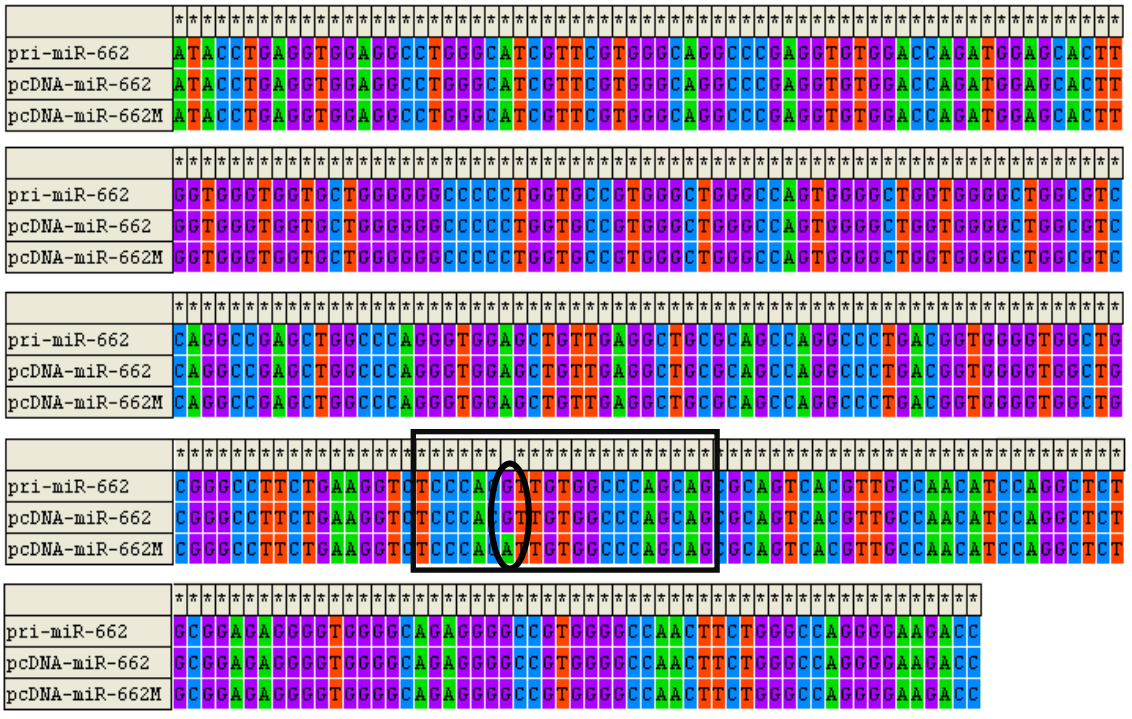
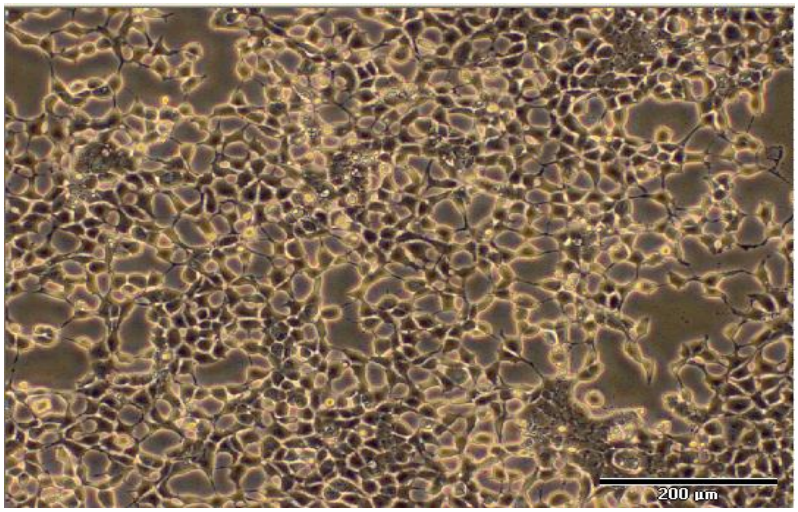


Figure 3-16: Sequence alignment of pri-miR-662 from miRBase database, recombinant plasmid of pri-miR-662 (pcDNA-miR-662) and recombinant plasmid of mutagenized pri-miR-662 (pcDNA-miR-662M).

3.10 Cell culture

pEGFP vector acted as a control to check the efficiency of transfection. The pEGFP vector (Clontech, United States) was transfected along with wild-type and variant of pri-miRNA gene in a six-well plate. The green fluorescence signals emitted from transfected HEK 293T cells were captured through fluorescent microscope. From cell count, the rate of transfection was more than 75% after comparing green fluorescent cells to the total HEK293T cells captured from light microscope. This showed good transfection efficiency (Figure 3-17).

i)



ii)

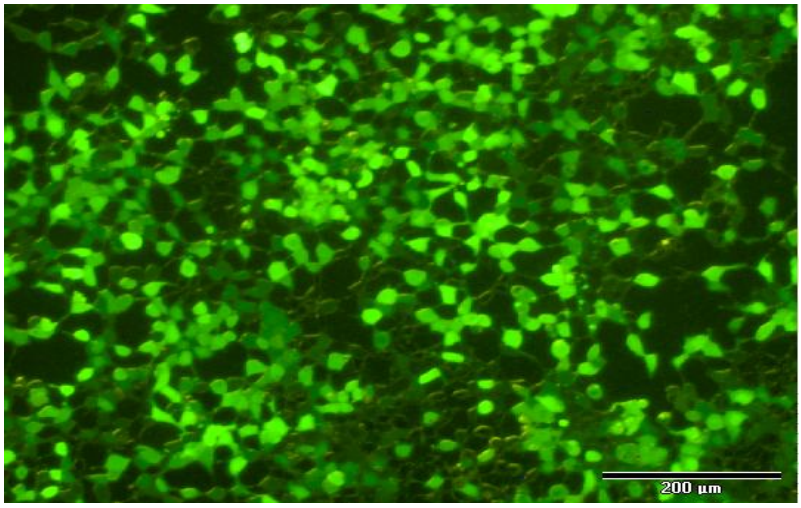


Figure 3-17: Transfection rate was checked by comparing on the same spot of cells under microscope.
(i) HEK 293T cells captured under light microscope and (ii) Green fluorescence cells that were captured thought fluorescent microscope.

3.11 RNA extraction from cell culture

Total RNA was isolated from five transfected HEK 293T cells with the acid guanidine isothiocyanate-phenol: chloroform (as mentioned in section 2.2.6.1). RNA samples were visualized on a 1% agarose gel (Figure 3-18) and visible bands were observed in each well corresponding to expected sizes for ribosomal RNA (rRNA): 18S and 28S rRNA. The RNA extracted appeared to have good quality and without any sign of degradation. The A_{260}/A_{280} ratio from spectrophotometer was approximately 2.0, which proved that the extracted RNA was clean.

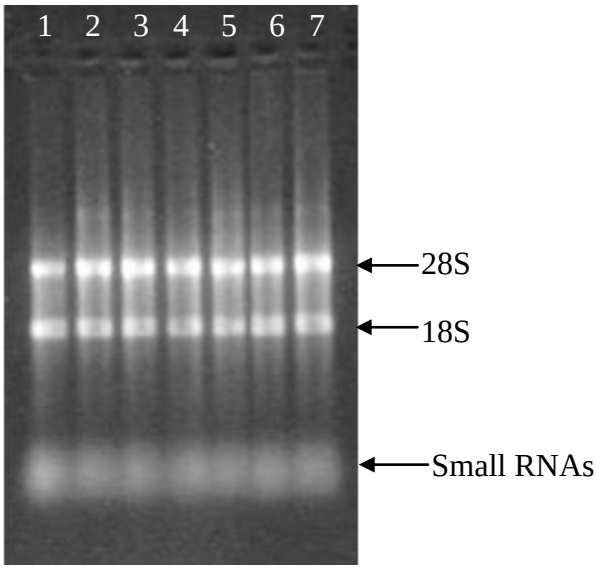


Figure 3-18: Agarose gel of total RNA samples extracted from HEK 293T cells.
Lane 1 is empty vector of pcDNA^{TM} 3.1, lane 2 is from wild-type pri-miR-125a pcDNA plasmid, lane 3 is mutagenized pri-miR-125a pcDNA plasmid, lane 4 is wild-type pri-miR-124-3 pcDNA plasmid, lane 5 is mutagenized pri-miR-124-3 pcDNA plasmid, lane 6 is wild type pri-miR-662 pcDNA plasmid and lane 7 is mutagenized pri-miR-662 pcDNA plasmid.

3.12 Statistical analysis of quantitative real time polymerase chain reaction (qRTPCR)

3.12.1 TaqMan miRNA assay for mature miRNAs

All two mature miRNA expression levels (miR-125a, miR-124-3 and miR-662) in transfected HEK 293T cells were quantified by using TaqMan miRNA Assay. This is because TaqMan miRNA probe has high specificity (Chen *et al*, 2005). Results generated from ABI SDS system was analyzed using Microsoft Excel. MicroRNA expression data is presented as fold difference relative to the mean between RNU44 and miR-16 based on the following equation: relative quantification, $RQ = 2^{-\Delta\Delta CT}$. A two-dimension column was plotted to illustrate the results.

Mature miRNA expression level was normalized to endogenous controls to obtain ΔC_T values, and the fold difference is relative to the calibrator (empty vector) to obtain $\Delta\Delta C_T$ values. The relative quantification (RQ) of mature miRNA was calculated by applying to the $2^{-\Delta\Delta CT}$ analyses. RQ value was converted to Log unit to better represent the data.

Single nucleotide variations within seed region of mature miRNA sequence showed different expression level compared to the wild-type gene of miRNAs (Figure 3-19). For the positive control (miR-125a) in this study, expression level of mutagenized allele miR-125a was upregulated 1.7 fold, $p_{t\text{-}test} = 1.12 \times 10^{-12}$, compared to the wild-type allele miR-125a. The expression level of mutagenized allele of miR-124-3 and mutagenized allele of miR-662 were both upregulated compared to the wild-type allele of miR-124-3 and wild type allele of miR-662 respectively. Mutagenized allele of miR-124-3 was upregulated 2.2 fold, $p_{t\text{-}test} = 5.16 \times 10^{-7}$, while mutagenized allele of miR-662 was upregualted 1.9 fold, $p_{t\text{-}test} = 4.55 \times 10^{-4}$ (refer to Apendix).

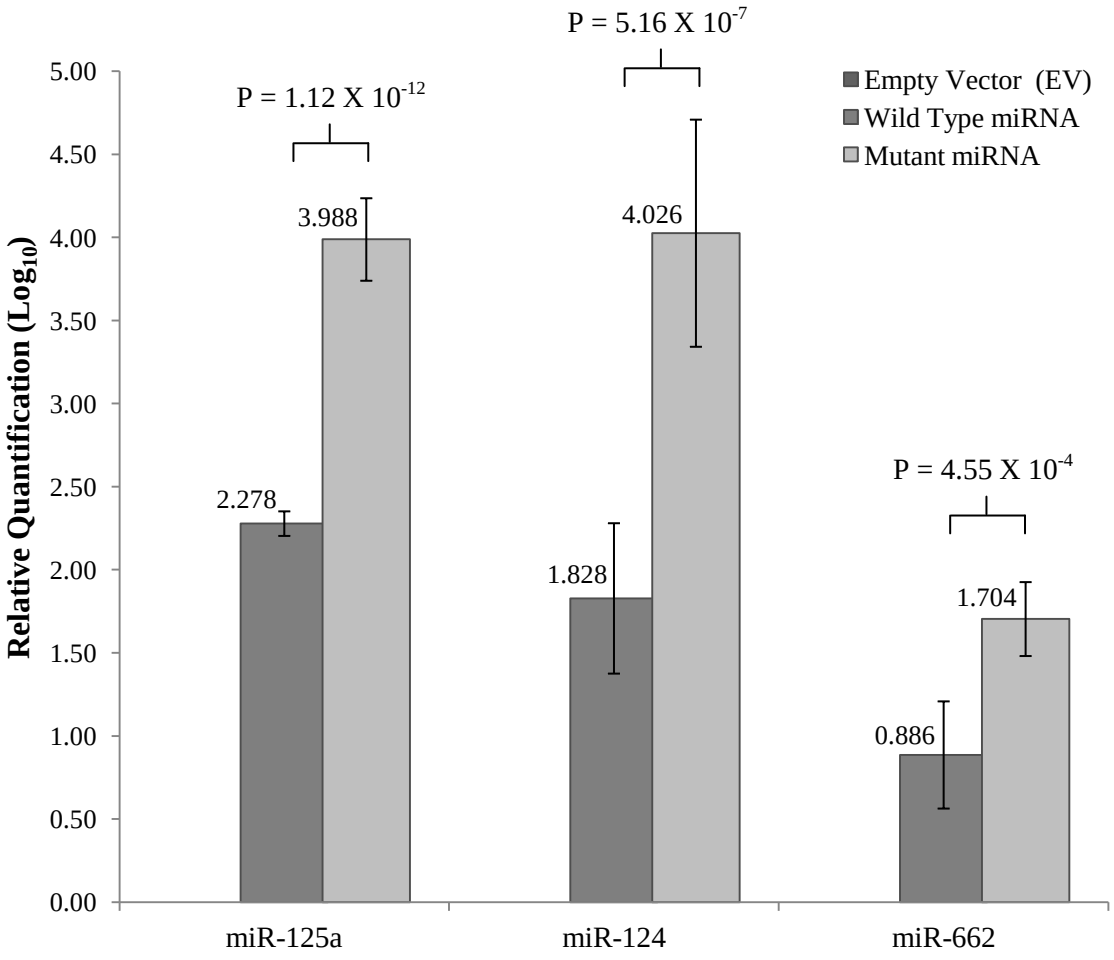


Figure 3-19: Comparision of relative quantification between miR-125a, miR-124-3 and miR-662.
Relative expression level of mature miR-125a, miR-124-3 and miR-662 normalized to both RNU44 and miR-16 (endogenous controls). The relative expression levels were determined by ΔΔCT analyses. Transfection was repeated three times. $P_{t\text{-}test} < 0.05$.

3.12.2 Expression level of mutagenized miRNA gene relative to wild-type miRNA gene in percentage (%)

The expression level of both wild-type and mutagenized pri-miRNAs and pre-miRNAs were determined through SYBR Green Real Time PCR by using specific primers. Pri-miRNA and pre-miRNA expression level were normalized to internal controls (GAPDH and beta-actin) to obtain ΔC_T values and relative to the calibrator (empty vector) to obtain $\Delta\Delta C_T$ values. Relative expression levels of pri-miRNA (PRI), pri- and pre-miRNA (PRI + PRE) and mature miRNAs (miRNA) were plotted in two-dimension column. The expression level of miRNA (pri, pri + pre and mature miRNA)

was quantitated by obtaining the ratio between mutagenized miRNA to wild-type miRNA.

The results showed that the primary transcript of mutagenized miR-125a was increased by 16% when compared with wild-type miR-125a gene, while mutagenized pri- and pre-miR125a expression level was increased by 19%. However, mutagenized mature miR-125a expression level was 75% upregulated when compared to the wild-type miR-125a gene (Figure 3-20 A).

Chart in Figure 3-20 (B) showed that the expression level of mutagenized pri-miR-124-3 was increased slightly by 12% compared to the wild-type pri-miR-124-3. For the expression level of mutagenized pri- and pre-miR-124-3, the chart showed that approximately 8% of increment after comparison with the wild-type gene. Meanwhile, the expression of mutagenized miR-124-3 in HEK 293T cells was increased to about 120 % of wild-type miR-124-3.

In Figure 3-20 (C), the expression of mutagenized pri-miR-662 was showed to be the same as the wild type of pri-miR662, while the expression of mutagenized pri- and pre-miR-662 was slightly inhibited by 13%. On the other hand, the expression of mature form of mutagenized miR-662 was improved, 92% that of the wild-type allele of miR-662.

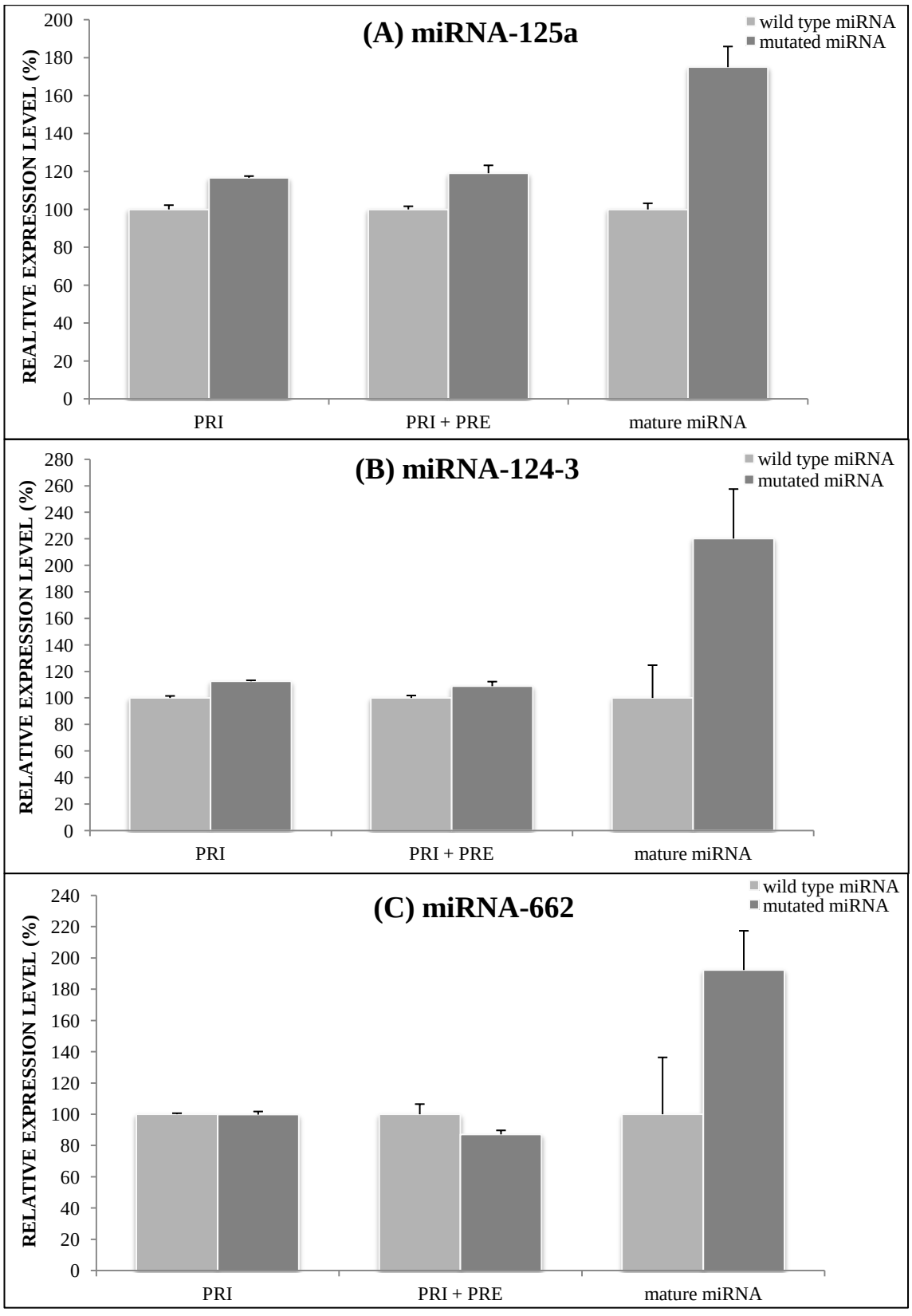


Figure 3-20: Quantitative Real Time PCR was used to measure the levels of pri-, pre- and mature forms of wild-type miRNA and mutagenized miRNA.
(A) miR-125a/ miR-125aM, (B) miR-124-3/miR-124-3M, (C) miR-662/miR-662M, in the transfected cells. The relative expression levels were determined by ΔΔCT analyses. Three individual technical repeats were performed and each transfection was in duplicates.

CHAPTER 4 DISCUSSION

4.1 Features of miRNA function

MicroRNA, a group of small non-coding RNAs is known to play important roles in biological functions by regulating gene expression through translation inhibition. MicroRNAs was known to be involved in cell developmental (Ambros, 2004; Kim and Nam, 2006; Kusenda *et al*., 2006; Meola *et al*., 2009), cell proliferation, apoptosis, progression of human diseases (Lai, 2003; Ambros, 2004; Bartel, 2004; Hammond, 2006; Cho, 2007), determination of timing and maintenance of tissue or cell identity (Zeng and Cullen, 2003; Ambros, 2004; Bartel, 2004; Zhang *et al*., 2006) and also in responding to environmental stresses, immune system, and neurological processes (Bartel, 2004; Du and Zamore, 2005; Wiemer, 2007; Garofalo *et al*., 2008; Meola *et al*., 2009). Hypothetically, sequence variation within miRNA genes (pri-miRNA, pre-miRNA or mature miRNA) would potentially affect the miRNA processing and/or 3' UTR mRNA target selection (Iwai and Naraba, 2005; Shen and Yu, 2008; Wu *et al*., 2008; Mencía *et al*., 2009; Sun *et al*., 2010). The expression profiles of miRNAs in human cancers and disorders had been extensively studied over the past decades. Many miRNAs were up- or down-regulated in various human cancers and disorder. This indicates that miRNAs play a role in tumorigenesis. However, compared with the comprehensive studies of miRNA expression profiling, associations between miRNA mutations with cancers or disorders are still larger unknown (Iwai and Naraba, 2005; Shen and Yu, 2008; Wu *et al*., 2008).

4.2 Genetic variation of miRNA sequences

Data mining was performed to further investigate the functional role of miRNAs, by searching for homology between 1344 known miRNAs in human genome (from miRBase Release 16.0, Sept 2010) and SNPs database (from dbSNP131/132, UCSC Genome browser). A total amount of 42 SNPs in seed-region of mature miRNA were identified. These findings can conclude that miRNA genes have very low polymorphism density since most identified variations are not within the seed region, indicating pre-miRNAs have a strong selective constraint (Borel and Antonarakis, 2008). These single nucleotide variant within seed region sequence of mature miRNAs could have complex influence by affecting miRNA biogenesis, functional strand selection and target selection. This study focused on the genetic variation within the seed region of 5'-end mature miRNA, rather than pri-miRNA or pre-miRNA because seed region base pairing is known to be a critical region for 3'UTR mRNA target recognition (Bartel, 2004; Ambros, 2004; Lee *et al*., 2006; Zambon *et al*., 2006). This would result in targets gain or loss. Theoretically, functional mutation found on the seed region would hugely impact mRNA-protein translation, further contributing to disease or cancer development.

The RNA secondary structure of these 42 single nucleotide variant within seed region sequence of mature miRNAs was predicted by MFOLD program to examine how the variant allele could alter the secondary structure of related wild-type allele of miRNAs. Only 14 single nucleotide variant within seed region sequence of mature miRNAs showed base-pairing mismatches, increased minimal free energy values and the addition of an enlarged RNA bulge in the predicted secondary structure (as shown in Figure 3.1). Among these potential single nucleotide variants, 12 single nucleotide variant on the seed region of mature miRNAs were not available when this project was first conducted, as the miRBase database used was Release 13.0, March 2009. New

additional database containing these 12 single nucleotide variant were only available on Release 19.0, Aug 2012. They are miR-3117, miR-3118-2, miR-3118-3, miR-3124, miR-3128, miR-3161, miR-3618, miR-3689b, miR-3910-2, miR-3939, miR-4257 and miR-4293. From the remaining single nucleotide variant within seed region sequence of mature miRNAs (miR-124-3, miR-125a, miR-662, miR-941-1 and miR-1276), miR-124-3 variant (miR-124-3M) and miR-662 variant (miR-662M) were chosen as the study subjects. miR-125a was used as the positive control throughout the experiment, since study on miR-125a had been carried by Duan and his colleague (year 2007).

Currently, no population study for miR-125a (SNP ID: rs12975333) and miR-124-3 variant (SNP ID: rs 34059726) has been conducted and therefore no allele frequency data is available. The variation observed in miR-124-3 was a transversional substitution, with Guanine as the major allele, Thymine as the minor allele. Whereas for miR-662 (SNP ID: rs 9745376), association study was performed in the population of Bushman, European, Bantu and Nigeria (Saunders *et al*., 2007), Table 4.1. The variation in miR-662 variant was a transitional substitution, with Guanine as the major allele, Adenine as the minor allele. The 1000 Genomes project had reported the global minor allele frequency of this SNP (rs 9745376) to be 0.029.

Table 4.1: Allele frequency of miR-662 in 4 populations (overall 178 individuals)

Populations	**Allele Frequency**	
	Allele A (Wild Type)	**Allele G (Variant)**
Bushman	0.667	0.333
European	0	1.000
Bantu	1.000	0
Nigeria	0.140	0.860

4.3 Pre-miRNA secondary structure prediction

The selected secondary structure of two wild-type and variant allele of pre-miRNAs showed increament in the negative minimal folding energy value, showed in Table 4.2.

Table 4.2: Minimal folding energy (MFE) value of miRNA secondary structure.

microRNA	**Minimal Folding Energy (MFE) Value**		**Energy Change (ΔΔG)**
	Wild Type Allele	**Variant Allele**	
miR-125a	-46.1kcal/mol	-40.0kcal/mol	6.1 kcal/mol
miR-124-3	-40.30kcal/mol	-32.20kcal/mol	8.1 kcal/mol
miR-662	-47.40kcal/mol	-46.30kcal/mol	1.1 kcal/mol

All pre-miRNAs containing variant allele showed less negative minimal folding energy compared to their wild-type pre-miRNA. Since stem looped secondary structures of miRNAs are well conserved in humans (Ambros *et al*., 2003; Kim and Nam, 2006; Zhang *et al*., 2006), every wild-type miRNA should has a unique secondary structure and the minimum free energy value represent the stable folding of secondary structure of one specific miRNA type. Deviation of the minimal folding energy value might affect the initiate stable structure of miRNA and hence alter the production of mature miRNA. These pre-miRNAs with variant allele seemed to strengthen the stability of the secondary structure. Hence, single nucleotide variantion within seed region sequence of mature miR124-3 and miR-662 also have the potential to influence the miRNA gene transcription, miRNA processing or the mRNA targeting.

4.4 Validation of wild-type and variant allele in two miRNA forms

4.4.1 Single nucleotide variant in seed-region miR-124-3 and miR-662 enhanced the processing of mature miRNA

Study from Duan and colleagues (2007) demonstrated that mutation at seed region of miR-125a, can reduced the expression level of pre- and mature miR-125a that containing the variant allele. Nucleotide variation at the eighth nucleotide upstream from the 5' end of the mature miR-125a has proven to have a huge impact on Drosha-DGCR8 processing of its RNA substrate (Duan *et al*., 2007). In my findings, the mature miR-125a showed an enhanced expression level which is different from Duan's finding. This could possibly due to the different vector and cell line used. In my study, pcDNA TM 3.1/Zeo (-) vector with a Cytomegalovirus (CMV) enhancer-promoter from Invitrogen was used instead of pSM2 retroviral vector with u6 promoter from Open Biosystems. Additionally, HEK293T transient cell line was used in this study rather than the stable HEK293. HEK293T contained T-antigen SV40 that enhanced the amplification of transfected plasmid, increasing the transfection efficiency (Oka *et al.*, 2010).

I found that G/T transversion at the fourth nucleotide upstream from the 5' end of the mature miR-124-3 and G/A transition at the seventh nucleotide upstream from the 5' end of the mature miR-662 had enhanced expression of the mature miRNA variant. The variations that occurred at seed region within mature miR-124-3 and miR-662 had increased the expression level of mature miRNA variant, they may provide a more stable stem preceding the mature miRNA sequence through some unknown molecular event (Sun *et al*., 2010). The structural features of these two pre-miRNAs may have stabilized the enzyme-substrate complex by mediating protein-nucleic acid interactions between Dicer and its RNA substrate. Dicer itself has the intrinsic ability to differentiate

RNA substrates with different structure (Chakravarthy *et al.*, 2010), since their cutting sites are secondary structure dependent and not sequence based (Sun *et al*., 2010). Therefore a stable enzyme-RNA substrate complex is believed to promote the Dicer-mediated processing of mature miRNA (Chakravarthy *et al.*, 2010). This could explain the 2.2 fold increase in production of mature miR-124-3 variant and 1.9 fold increase for mature miRNA of miR-662 variant. Further investigations such as Northern blot and reporter assay should be conducted to reconfirm these miRNAs that enhance processing of mature miRNA. It would be interesting to determine how the single nucleotide variation in the seed region of the miRNAs studied lead to increased expression of the miRNA variant.

4.4.2 Single nucleotide variation in seed-region miR-124-3 and miR-662 do not affect pri- and pre-miRNA processing

The expression level of variant allele of pre-miRNA for miR-124-3 and miR-662 remains almost similar to the wild-type allele of pre-miRNA expression. This showed that the variant allele on seed region on the 5'-end of mature miR-124-3 and miR-662 did not affect the processing of pre-miRNA from pri-miRNA. This result was different from the finding by Duan and his colleague, which stated that SNP on seed region could reduce the expression of pre- and mature miRNA. This outcome could be due to the different position of the variation site in the miRNA that affect the recognition site of complex protein. Many studies have reported that pri-miRNA is processed by Drosha-DGCR8 complex to produce pre-miRNA (Han *et al*., 2006; Yeom *et al*., 2006; Duan *et al*., 2007; Kunej *et al.*, 2012; Slaby *et al.*, 2012). Pre-miRNA was produced through the cleavage of approximately 22nt from the terminal stem-ssRNA junction by Drosha (Iwai and Naraba, 2005). If mutation causes a structural change or disrupt the stem integrity within this critical region of miRNA, processing and expression of miRNA

would be affected (Iwai and Naraba, 2005; Wu *et al*., 2008). Drosha-DGCR8 complex protein may bind to the pri-miRNA in one of two different orientations: productive processing (processing center is located ~11bp from the basal segment) and abortive processing (processing center is located ~11bp from the terminal loop), showed in Figure 4-1 (Han *et al*., 2006). Productive processing will produces functional pre-miRNA, while abortive processing product does not contain full miRNA sequences. Functional pre-miRNAs are favourably produced in the productive orientation. Han and colleagues suggested that internal stem structure may influenced the choice of processing. If a bulge was found to be near to the productive site, productive processing will be suppressed and abortive processing will be facilitated (Han *et al*., 2006).

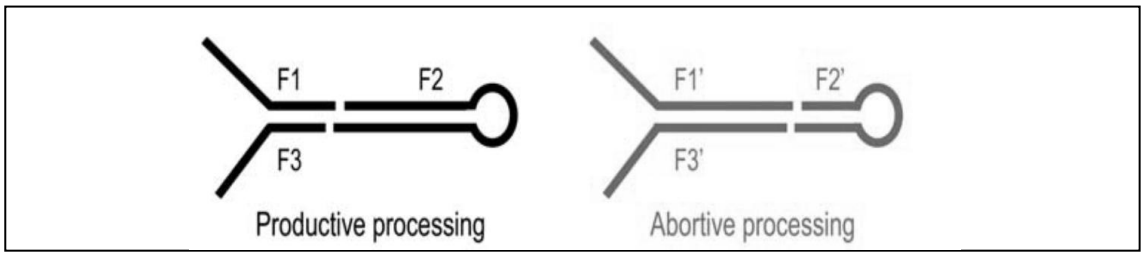


Figure 4-1: Productive Processing versus Abortive Processing. The fragment from abortive processing are designated as F1′, F2′, and F3′ (Figure from Han et al., 2006).

In this study, the variant allele that introduced the RNA bulge was located on the 32th nt from the basal segment (3'-end) of miR-124-3 and on the 29th nt from the basal segment (3'-end) of miR-662; both variant alleles are slightly far away from the 'ssRNA–dsRNA junction-anchoring' structure. The additional bulges are located close to the abortive processing site, which highly reduced the chance for abortive processing and facilated the productive processing which will produced the pre-miRNAs normally. Besides that, variant allele that were introduced in miR-124-3 and miR-662 did not affect the structure of ssRNA-dsRNA junction and hence do not affect the Drosha-

DGCR8 cutting site. Drosha excision sites are structure based and not sequence based (Sun et al., 2010). Therefore, pre-miR-124-3 and miR-662 was normally expressed.

4.5 Relationship between microRNA expression and disease predisposition

MicroRNAs play an essential role in many biological functions. The synergistic effects of individual miRNAs that can bind to the same mRNA target enable a miRNA family the precise modulation of target gene expression (Lee *et al*., 2006; Zhang *et al*., 2006; Sun *et al*., 2010).

Function of miR-124-3:

The mature miR124 family consists of miR-124-1, miR-124-2 and miR-124-3. In this study, only miR-124-3 show sequence variation on its seed region. miR-124-3 (MI0000445) is located in chromosome 20q13.33, region 61809907-61809907; all of which contain CpG islands in their promoter regions. The 3'-UTR target messenger RNA (mRNA) for miR-124-3 was predicted through mining the public database – PicTar (http://pictar.mdc-berlin.de), TargetScan (www.targetscan.org/) and Diana-Mirco lab (http://diana.cslab.ece.ntua.gr/microT/) programs. From the list of potential mRNA targets that were convergent among the three databases, only seven mRNA genes were ramdomly selected (Table 4.4): VAMP3 (Vesicle-associated membrane protein 3), CD164 (Sialomucin core protein 24), PTPN12 (Tyrosine-protein phosphatase non-receptor type 12), NEGR1 (Neuronal growth regulator 1), CDK6 (Cyclin-dependent kinase 6), ITGB1 (Integrin Beta 1) and IGFBP7 (Insulin-like growth factor-binding protein 7). Some of these target mRNAs had been experimentally verified.

For example, miR-124 expression led to the down-regulation of endogenous PTPN12 (protein tyrosine phosphatase non-receptor type 12) protein levels. PTPN12 regulate the equilibrium of tyrosine phosphorylation that plays a central role in cellular physiology and in cancer. PTPN12 also suppresses mammary epithelial cell proliferation and transformation. This suggests that miR-124 may mediate tumor suppressor RE1 silencing transcription factor's (REST) ability to inhibit PTPN12 protein expression. The inhibition of PTPTN12 tumor suppressor by miR-124 showed that miR-124 may function as an oncogene in human breast cancers and lung cancers (Sun *et al*., 2011). However in this study, miR-124-3 with a variant allele was predicted to target CREB5 (Cyclic AMP-responsive element-binding protein 5) and DCC (a novel gene, Deleted in Colorectal Cancer), instead of PTPN12 (predicted by public databases). This showed that the variant miR-124-3 may not be able to inhibit PTPTN12 tumor suppressor. Variant miR-124-3 may inversely act as tumor suppressor instead of oncogene in human breast cancers or lung cancers. On the other hand, variant miR-124-3 may target CREB5 gene and the up-regulation of CREB5 gene would cause HIV Encephalitis.

Besides that, Hunt and his colleagues had proven that miR-124 was able to suppress oral squamous cell carcinoma (OSCC) invasion by decreasing the ITGB1 expression level. ITGB1 is known to maintain tissue homeostasis and direct regulatory intracellular signaling cascades. From their study, mir-124 showed great potential as a prognostic indicator in OSCC (Hunt *et al.*, 2011).

Function of miR-662: Mature miR-662 (MI0003670) is located at chromosome 16p13.3, position 820183-820277. The 3'-UTR target mRNA for miR-662 was identified using public database – TargetScan (www.targetscan.org/) and Diana-Mirco lab (http://diana.cslab.ece.ntua.gr/microT/). Among the potential targets, only those that were convergent among the two databases were selected (Table 4.3): NEGR1 (Neuronal growth regulator 1), CSF3 (colony stimulating factor 3) and MKX (Homeobox protein Mohawk). There is currently no studies done on miR-662, therefore the target mRNAs were not validated. Enfield and his colleagues (2011) had proven that miR-662 transiently increased in response to high doses of X-ray radiation in human fibroblasts of lung cancer and may have a role in conferring chemoresistance to a number of lung cancer drugs (Enfield *et al.*, 2011). The expression of miR-662 was shown to increase in human kidney tissue of lupus nephritis patient (Dai *et al.*, 2009, Dalal and Kwon, 2010). Overall, miR-662 has a role in malignancies or in the control of growth and apoptosis (Dahiya *et al.*, 2008).

Table 4.3: Predicted potential 3' UTR mRNA targets for miR-124-3 and miR-662

miRNAs	Predicted mRNA target	Functions	Possible diseases or cancers
Wild Type miR-124-3	VAMP3	Vesicular transport from the late endosomes to the trans-Golgi network	Immunological disease
	CD164	Promotes myogenesis by enhancing CXCR4-dependent cell motility.	↑ Prostate cancer
	PTPN12	Dephosphorylates cellular tyrosine kinases	↓ Breast and lung cancer
	NEGR1	Cell-adhesion	↓ Ovarian cancer
	CDK6	Control of the cell cycle and differentiation; promotes G1/S transition	↓ Breast cancer
	ITGB1	Receptors for collagen	↓ Oral squamous cell carcinoma
	IGFBP7	Stimulates cell adhesion	↑ Cervical cancer
Variant miR-124-3	CREB5	Binds to the cAMP response element and activates transcription	↑ HIV Encephalitis (HIVE)
	DCC	Receptor for netrin required for axon guidance.	↑ Colorectal cancer
Wild Type miR-662	NEGR1	cell-adhesion	↓ Ovarian cancer
	CSF3	Induces granulocytes	↓ Smoking-induced chronic obstructive pulmonary disease.
	MKX	Morphogenetic regulator of cell adhesion	↑ Ovarian cancer
Variant miR-662	Zinc finger proteins	Transcriptional regulation	↑ pancreatic carcinomas ↓ Colorectal cancer

Annotations: VAMP3(Vesicle-associated membrane protein 3), CD164 (Sialomucin core protein 24), PTPN12 (Tyrosine-protein phosphatase non-receptor type 12), NEGR1 (Neuronal growth regulator 1), CDK6 (Cyclin-dependent kinase 6), ITGB1 (Integrin Beta 1), IGFBP7 (Insulin-like growth factor-binding protein 7), CREB5(Cyclic AMP-responsive element-binding protein 5), DCC(Deleted in Colorectal Cancer), NEGR1(Neuronal growth regulator 1), CSF3(colony stimulating factor 3) and MKX(Homeobox protein Mohawk).

4.6 Future aspects

Single nucleotide changes in the mature miRNA sequence may drive the evolution of new miRNA by altering their biological function. As nucleotide variation on seed region of miRNA genes are rare and their mutant allele frequencies are low, large studies will be needed to draw out the importance. Up-to-date, not many SNP information were available on the non-coding gene, where miRNAs genes are deposited. In additional, the complexity of the miRNA network, especially their pathways, can further intensified by the discovery of miRNA functions that mediated by changes in miRNA sequence and maturation. In order to gain accurate quantitative result and a convicing statistic data, usage of pri- and pre-miRNA Taqman probes would gives high specificity to the target gene and reliable result as compare to SYBR Green probe.

The findings of these two single nucleotide variations of miRNAs can be used to further study the effect of genetic variation on one population risk of cancer or disorders. In future, Western blot, reporter assay and luciferase assay could be used to study the linkage between alterations in miRNA structure and the predicted target mRNAs and cancer risk. The study of genetic variation in miRNA networks has expanded our knowledge of the possible ways in which miRNAs can affect cancer.

CHAPTER 5 CONCLUSION

The evolutionary conservation of mature miRNA genes within humans allows bioinformatic approaches to be used to predict the potential miRNA variations against the Single Nucleotide Polymorphism database. Among the 1344 mature miRNA sequences, 42 single nucleotide variants were found on the seed region of mature miRNA by using BLAT homology search. Two selected potential single nucleotide variation in seed-region of mature miRNAs had been experimentally validated for the pri-, pre- and mature miRNA expression by real-time PCR approach. These two single nucleotide variations in seed-region of mature miRNAs that had an additional RNA bulge, located slight further from the 5'-end of mature miRNA surprisingly provided a more stable structure of pri-miRNA and pre-miRNA. This outcome in return increases the expression of mature miRNAs. MicroRNAs are known to be important for gene regulation network by regulating gene expression at the post transcriptional stage. Therefore, the study of sequence variation on seed-region of miRNAs could enhance the understanding of miRNA biogenesis and the potential contribution of these nucleotide variations to normal human variation and disease pathogenesis. However, single nucleotide variations in seed-region mature miRNA that was associated to cancer development is very much in its infancy and hence further investigation on mRNA targeting is necessary. Future studies could include analyzing potential direct targets predicted by bioinformatics programs and assessing gene-silencing capabilities of both mutants through luciferase assay. In addition, association studies on cancer prone populations can be done to elucidate the frequency of miRNA allele polymorphisms and its effect on cancers development.

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APPENDIX

Table A.1: Homology search (BLAT) of 127 potential *Homo sapiens* single nucleotide variation on mature miRNA gene.

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
1	hsa-mir-92a-1 MI0000093	AGGTTGGGATCGGTTGCAATGCT	rs72631821	chr13:92003588-92003588	+	C/T	
			rs9589207	chr13:92003589-92003589	+	A/G	
2	hsa-mir-105-2 MI0000112	TCAAATGCTCAGACTCCTGTGGT	rs72631816	chrX:151562938-151562938	-	A/T	
3	hsa-mir-34a MI0000268	TGGCAGTGTCTTAGCTGGTTGT	rs35301225	chr1:9211802-9211802	+	A/C/T	
4	hsa-mir-7-1 MI0000263	CAACAAATCACAGTCTGCCATA	rs76662330	chr9:86584707-86584707	+	G/T	
5	hsa-mir-183 MI0000273	TATGGCACTGGTAGAATTCACT	rs41281222	chr7:129414806-129414806	+	C/T	
6	hsa-mir-196a-2 MI0000279	CGGCAACAAGAAACTGCCTGAG	rs11614913	chr12:54385599-54385599	+	C/T	
7	hsa-mir-204 MI0000284	TTCCCTTTGTCATCCTATGCCT	rs112062096	chr9:73424956-73424956	+	A/G	
8	hsa-mir-221 MI0000298	ACCTGGCATACAATGTAGATTT AGCTACATTGTCTGCTGGGTTT	rs113054794	chrX:45605666-45605666	+	A/C	seed
9	hsa-mir-122 MI0000442	TGGAGTGTGACAATGGTGTTTG AACGCCATTATCACACTAAATA	rs41292412	chr18:56118358-56118358	+	C/T	seed
10	hsa-mir-124-3 MI0000445	CGTGTTCACAGCGGACCTTGAT TAAGGCACGCGGTGAATGCC	rs34059726	chr20:61809907-61809907	+	G/T	seed
11	hsa-mir-125a MI0000469	TCCCTGAGACCCTTTAACCTGTGA ACAGGTGAGGTTCTTGGGAGCC	rs12975333	chr19:56888340-56888340	+	G/T	seed
12	hsa-mir-146a MI0000477	TGAGAACTGAATTCCATGGGTT CCTCTGAAATTCAGTTCTTCAG	rs2910164	chr5:159844996-159844996	+	C/G	seed
13	hsa-mir-154 MI0000480	TAGGTTATCCGTGTTGCCTTCG AATCATACACGGTTGACCTATT	rs41286570	chr14:100595880-100595880	+	A/G	
14	hsa-mir-299 MI0000744	TGGTTTACCGTCCCACAT TATGTGGGATGGTAAACCGCTT	rs41286566	chr14:100559898-100559898	+	C/T	
15	hsa-mir-302b MI0000772	ACTTTAACATGGAAGTGCTTTC TAAGTGCTTCCATGTTTTAGTAG	rs114318553	chr4:113569695-113569695	+	G/T	
16	hsa-mir-383 MI0000791	AGATCAGAAGGTGATTGTGGCT	rs112302475	chr8:14711013-14711013	+	G/T	
17	hsa-mir-412 MI0002464	ACTTCACCTGGTCCACTAGCCGT	rs61992671	chr14:101531854-101531854	+	A/G	
18	hsa-mir-373 MI0000781	ACTCAAAATGGGGGCGCTTTCC GAAGTGCTTCGATTTTGGGGTGT	rs80338016	chr19:54292016-54292016	+	C/T	

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
19	hsa-mir-379 MI0000787	TGGTAGACTATGGAACGTAGG TATGTAACATGGTCCACTAACT	rs61991156	chr14:101488414-101488414	+	A/G	seed
			rs72631818	chr14:101488424-101488424	+	A/G	
20	hsa-mir-148b MI0000811	AAGTTCTGTTATACACTCAGGC TCAGTGCATCACAGAACTTTGT	rs74878365	chr12:54731071-54731071	+	A/C	
21	hsa-mir-339 MI0000815	TCCCTGTCCTCCAGGAGCTCACG TGAGCGCCTCGACGACAGAGCCG	rs72631820	chr7:1062599-1062599	-	A/G	
22	hsa-mir-431 MI0001721	TGTCTTGCAGGCCGTCATGCA CAGGTCGTCTTGCAGGGCTTCT	rs12884005	chr14:101347408-101347408	+	A/G	seed
23	hsa-mir-485 MI0002469	AGAGGCTGGCCGTGATGAATTC GTCATACACGGCTCTCCTCTCT	rs112982830	chr14:101521764-101521764	+	A/G	
24	hsa-mir-523 MI0003153	CTCTAGAGGGAAGCGCTTTCTG GAACGCGCTTCCCTATAGAGGGT	rs112495387	chr19:54201668-54201668	+	A/G	
25	hsa-mir-520c MI0003158	CTCTAGAGGGAAGCACTTTCTG AAAGTGCTTCCTTTTAGAGGGT	rs7255628	chr19:54210734-54210734	+	C/G	
26	hsa-mir-519d MI0003162	CAAAGTGCCTCCCTTTAGAGT	rs116796400	chr19:54216670-54216670	+	A/G	
27	hsa-mir-518e MI0003169	CTCTAGAGGGAAGCGCTTTCTG AAAGCGCTTCCCTTCAGAG	rs74177813	chr19:54233109-54233109	+	-/C	seed
			rs34416818	chr19:54233113-54233112	+	-/A	seed
			rs74177814	chr19:54233114-54233113	+	-/G	seed
28	hsa-mir-518d MI0003171	CTCTAGAGGGAAGCACTTTCTG CAAAGCGCTTCCCTTTGGAGC	rs73602910	chr19:54238189-54238189	+	A/G	seed
29	hsa-mir-516b-1 MI0003172	ATCTGGAGGTAAGAAGCACTTT TGCTTCCTTTCAGAGGGT	rs78861479	chr19:54240174-54240174	+	A/G	
30	hsa-mir-499 MI0003183	TTAAGACTTGCAGTGATGTTT AACATCACAGCAAGTCTGTGCT	rs3746444	chr20:33578251-33578251	+	C/T	seed
31	hsa-mir-501 MI0003185	AATGCACCCGGGCAAGGATTCT	rs112489955	chrX:49774389-49774389	+	A/G	
32	hsa-mir-513a-1 MI0003191	TTCACAGGGAGGTGTCAT TAAATTTCACCTTTCTGAGAAGG	rs35027589	chrX:146295068-146295067	+	-/C	seed
33	hsa-mir-552 MI0003557	AACAGGTGACTGGTTAGACAA	rs76067517	chr1:35135270-35135270	+	A/G	
34	hsa-mir-92b MI0003560	AGGGACGGGACGCGGTGCAGTG TATTGCACTCGTCCCGGCCTCC	rs12759620	chr1:155165044-155165044	+	C/G	
35	hsa-mir-556 MI0003562	GATGAGCTCATTGTAATATGAG ATATTACCATTAGCTCATCTTT	rs77585320	chr1:162312411-162312411	+	C/T	

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
36	hsa-mir-557 MI0003563	GTTTGCACGGGTGGGCCTTGTCT	rs78825966	chr1:168344829-168344829	+	C/T	seed
37	hsa-mir-568 MI0003574	ATGTATAAATGTATACACAC	rs28632138	chr3:114035387-114035387	+	G/T	
38	hsa-mir-576 MI0003583	ATTCTAATTTCTCCACGTCTTT AAGATGTGGAAAAATTGGAATC	rs71603032	chr4:110409923-110409923	+	G/T	
39	hsa-mir-581 MI0003588	TCTTGTGTTCTCTAGATCAGT	rs1694089	chr5:53247394-53247394	-	G/T	
			rs810917	chr5:53247400-53247400	+	A/G	
40	hsa-mir-585 MI0003592	TGGGCGTATCTGTATGCTA	rs62376935	chr5:168690635-168690635	+	C/T	seed
41	hsa-mir-590 MI0003602	GAGCTTATTCATAAAAGTGCAG TAATTTTATGTATAAGCTAGT	rs6971711	chr7:73605599-73605599	+	C/T	
42	hsa-mir-593 MI0003605	AGGCACCAGCCAGGCATTGCTCAGC TGTCTCTGCTGGGGTTTCT	rs73721294	chr7:127721934-127721934	+	C/T	
43	hsa-mir-596 MI0003608	AAGCCTGCCCGGCTCCTCGGG	rs61388742	chr8:1765425-1765425	+	C/T	
44	hsa-mir-597 MI0003609	TGTGTGTCACTCGATGACCACTGT	rs112056940	chr8:9599212-9599212	+	C/T	
45	hsa-mir-605 MI0003618	TAAATCCCATGGTGCCTTCTCCT	rs113212828	chr10:53059349-53059349	+	A/G	seed
			rs76759855	chr10:53059356-53059356	+	A/G	
			rs34610391	chr10:77312285-77312284	+	-/A	
46	hsa-mir-608 MI0003621	AGGGGTGGTGTTGGGACAGCTCCGT	rs4919510	chr10:102734778-102734778	+	C/G	
47	hsa-mir-624 MI0003638	TAGTACCAGTACCTTGTGTTCA CACAAGGTATTGGTATTACCT	rs73251987	chr14:31483883-31483883	+	C/G	
48	hsa-mir-625 MI0003639	AGGGGGAAAGTTCTATAGTCC GACTATAGAACTTTCCCCCTCA	rs12894182	chr14:65937889-65937889	+	A/C	
49	hsa-mir-627 MI0003641	GTAGTGAGTCTCTAAGAAAAGAGGA	rs76362052	chr15:42491832-42491832	+	C/T	
			rs2620381	chr15:42491848-42491848	+	A/C	seed
50	hsa-mir-628 MI0003642	ATGCTGACATATTTACTAGAGG TCTAGTAAGAGTGGCAGTCGA	rs80185076	chr15:55665154-55665154	+	C/G	
51	hsa-mir-629 MI0003643	TGGGTTTACGTTGGGAGAACT	rs78212770	chr15:70371777-70371777	+	C/G	
52	hsa-mir-642a MI0003657	GTCCCTCTCCAAATGTGTCTTG	rs78902025	chr19:46178206-46178206	+	G/T	seed
53	hsa-mir-646 MI0003661	AAGCAGCTGCCTCTGAGGC	rs6513497	chr20:58883605-58883605	+	G/T	
54	hsa-mir-662 MI0003670	TCCCACGTTGTGGCCCAGCAG	rs9745376	chr16:820249-820249	+	A/G	seed
55	hsa-mir-449b MI0003673	AGGCAGTGTATTGTTAGCTGGC	rs10061133	chr5:54466544-54466544	+	A/G	
56	hsa-mir-208b MI0005570	ATATAAGACGAACAAAAGGTTTGT	rs2754157	chr14:23887219-23887219	+	A/T	

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
57	hsa-mir-449c MI0003823	TAGGCAGTGTATTGCTAGCGGCTGT TTGCTAGTTGCACTCCTCTCTGT	rs35770269	chr5:54468124-54468124	+	A/T	seed
58	hsa-mir-509-2 MI0005530	TACTGCAGACAGTGGCAATCA TGATTGGTACGTCTGTGGGTAG	rs36092315	chrX:146340339-146340339	+	-/T	
59	hsa-mir-933 MI0005755	TGTGCGCAGGGAGACCTCTCCC	rs79402775	chr2:176032376-176032376	+	A/G	
60	hsa-mir-936 MI0005758	ACAGTAGAGGGAGGAATCGCAG	rs79924817	chr10:105807928-105807928	+	C/T	seed
61	hsa-mir-938 MI0005760	TGCCCTTAAAGGTGAACCCAGT	rs12416605	chr10:29891260-29891260	+	C/T	seed
62	hsa-mir-940 MI0005762	AAGGCAGGGCCCCCGCTCCCC	rs35356504	chr16:2321820-2321820	+	-/C	
63	hsa-mir-941-2 MI0005764	CACCCGGCTGTGTGCACATGTGC	rs2427557	chr20:62551027-62551027	+	A/G	
			rs2427558	chr20:62551030-62551030	+	C/G	
64	hsa-mir-941-4 MI0005766	CACCCGGCTGTGTGCACATGTGC	rs76606291	chr20:62551216-62551216	+	A/G	seed
65	hsa-mir-944 MI0005769	AAATTATTGTACATCGGATGAG	rs77473380	chr3:189547778-189547778	+	C/T	
66	hsa-mir-297 MI0005775	ATGTATGTGTGCATGTGCATG	rs112636147	chr4:111781785-111781785	+	A/G	
67	hsa-mir-1200 MI0006332	CTCCTGAGCCATTCTGAGCCTC	rs77814495	chr7:36959015-36959015	+	A/G	
68	hsa-mir-1203 MI0006335	CCGGAGCCAGGATGCAGCTC	rs77574787	chr17:46233848-46233848	+	G/T	
69	hsa-mir-1304 MI0006371	TTTGAGGCTACAGTGAGATGTG	rs79759099	chr11:93466909-93466909	+	A/G	seed
			rs76857625	chr11:93466910-93466910	+	A/G	seed
70	hsa-mir-1291 MI0006353	TGGCCCTGACTGAAGACCAGCAGT	rs77994717	chr12:49048277-49048277	+	A/G	
71	hsa-mir-1299 MI0006359	TTCTGGAATCCTACGTGAGGGA	rs79965448	chr9:69002294-69002294	+	C/T	
			rs78546776	chr9:69002304-69002304	+	A/C/G	
			rs76266132	chr9:69002317-69002317	+	C/T	seed
72	hsa-mir-548l MI0006361	AAAAGTATTTGCGGGTTTTGTC	rs13447640	chr11:94199721-94199721	-	C/T	
73	hsa-mir-1268 MI0006405	CGGGCGTGGTGGTGGGGG	rs28599926	chr15:22513271-22513271	+	C/T	seed
74	hsa-mir-1269 MI0006406	CTGGACTGAGCCGTGCTACTGG	rs73239138	chr4:67142620-67142620	+	A/G	
75	hsa-mir-548h-3 MI0006413	AAAAGTAATCGCGGTTTTTGTC	rs9913045	chr17:13446924-13446924	+	A/G	
76	hsa-mir-548h-4 MI0006414	AAAAGTAATCGCGGTTTTTGTC	rs73235381	chr8:26906402-26906402	+	C/T	
77	hsa-mir-1275 MI0006415	GTGGGGGAGAGGCTGTC	rs77821659	chr6:33967796-33967796	+	A/C	
78	hsa-mir-1276 MI0006416	TAAAGAGCCCTGTGGAGACA	rs34381260	chr15:86313795-86313794	+	-/T	seed
79	hsa-mir-1255b-1 MI0006435	CGGATGAGCAAAGAAAGTGGTT	rs6841938	chr4:36428048-36428048	+	A/G	
80	hsa-mir-1321 MI0006652	CAGGGAGGTGAATGTGAT	rs36100620	chrX:85090853-85090852	+	-/G	
81	hsa-mir-1322 MI0006653	GATGATGCTGCTGATGCTG	rs59878596	chr8:10682894-10682894	+	C/T	

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
82	hsa-mir-1911 MI0008332	TGAGTACCGCCATGTCTGTTGGG	rs79202905	chrX:113997768-113997768	+	A/G	
83	hsa-mir-2053 MI0010487	GTGTTAATTAAACCTCTATTTAC	rs10505168	chr8:113655752-113655752	-	A/G	
84	hsa-mir-2277 MI0011284	AGCGCGGGCTGAGCGCTGCCAGTC TGACAGCGCCCTGCCTGGCTC	rs73772915	chr5:92956422-92956422	+	C/G	
85	hsa-mir-3117 MI0014130	ATAGGACTCATATAGTGCCAG	rs12402181	chr1:67094171-67094171	+	A/G	seed
86	hsa-mir-3118-2 MI0014132	TGTGACTGCATTATGAAAATTCT	rs11488501	chr1:143424165-143424165	+	C/T	seed
87	hsa-mir-3118-3 MI0014133	TGTGACTGCATTATGAAAATTCT	rs11488501	chr1:143424165-143424165	+	C/T	seed
88	hsa-mir-3124 MI0014140	TTCGCGGGCGAAGGCAAAGTC	rs12081872	chr1:249120584-249120584	+	C/T	seed
89	hsa-mir-3125 MI0014142	TAGAGGAAGCTGTGGAGAGA	rs33977954	chr2:12877503-12877502	+	-/A	
90	hsa-mir-3128 MI0014145	TCTGGCAAGTAAAAAACTCTCAT	rs61130794	chr2:178120681-178120681	+	G/T	seed
91	hsa-mir-3130-1 MI0014147	TACCCAGTCTCCGGTGCAGCC GCTGCACCGGAGACTGGGTAA	rs2241347	chr2:207647981-207647981	-	A/G	
92	hsa-mir-3152 MI0014179	TGTGTTAGAATAGGGGCAATAA	rs13299349	chr9:18573360-18573360	+	A/G	
93	hsa-mir-3130-2 MI0014148	TACCCAGTCTCCGGTGCAGCC GCTGCACCGGAGACTGGGTAA	rs2241347	chr2:207647981-207647981	-	A/G	
94	hsa-mir-548u MI0014168	CAAAGACTGCAATTACTTTTGCGC	rs2397267	chr6:57254986-57254986	+	A/G	
			rs2894843	chr6:57255001-57255001	+	A/C	
95	hsa-mir-3144 MI0014169	AGGGGACCAAAGAGATATATAG ATATACCTGTTCGGTCTCTTTA	rs67106263	chr6:120336384-120336384	+	A/G	
96	hsa-mir-3159 MI0014188	TAGGATTACAAGTGTCGGCCAC	rs79957895	chr11:18409354-18409354	+	A/G	
97	hsa-mir-3161 MI0014191	CTGATAAGAACAGAGGCCCAGAT	rs11382316	chr11:48118350-48118349	+	-/A	seed
			rs35834266	chr11:48118351-48118350	+	-/A	seed
			rs74581179	chr11:48118351-48118351	+	A/G	
99	hsa-mir-323b MI0014206	AGGTTGTCCGTGGTGAGTTCGCA CCCAATACACGGTCGACCTCTT	rs75330474	chr14:101522589-101522589	+	C/T	
100	hsa-mir-3175 MI0014209	CGGGGAGAGAACGCAGTGACGT	rs72647784	chr15:93447646-93447646	+	-/GA	
101	hsa-mir-3195 MI0014240	CGCGCCGGGCCCGGGTT	rs1886009	chr20:60639883-60639883	+	C/T	
102	hsa-mir-3196 MI0014241	CGGGGCGGCAGGGGCCTC	rs2273488	chr20:61870140-61870140	+	C/T	
103	hsa-mir-3198 MI0014246	GTGGAGTCCTGGGGAATGGAGA	rs56660112	chr22:18246960-18246960	+	C/G	
104	hsa-mir-4293 MI0015826	CAGCCTGACAGGAACAG	rs12220909	chr10:14425221-14425221	+	C/G	seed
105	hsa-mir-4302 MI0015833	CCAGTGTGGCTCAGCGAG	rs11048315	chr12:26026988-26026988	+	A/G/T	

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
106	hsa-mir-4257 MI0015856	CCAGAGGTGGGGACTGAG	rs74743733	chr1:150524468-150524468	+	A/G	seed
107	hsa-mir-4254 MI0015862	GCCTGGAGCTACTCCACCATCTC	rs12731294	chr1:32224285-32224285	+	G/T	
108	hsa-mir-4252 MI0015864	GGCCACTGAGTCAGCACCA	rs3982072	chr1:6489910-6489910	+	C/T	
			rs3982073	chr1:6489914-6489914	+	C/T	
109	hsa-mir-4265 MI0015869	CTGTGGGCTCAGCTCTGGG	rs4676066	chr2:109757963-109757963	+	C/T	
110	hsa-mir-4284 MI0015893	GGGGCTCACATCACCCCAT	rs11973069	chr7:73125664-73125664	+	C/T	
111	hsa-mir-3610 MI0016000	GAATCGGAAAGGAGGCGCCG	rs10094194	chr8:117886982-117886982	+	C/G	
112	hsa-mir-3618 MI0016008	TGTCTACATTAATGAAAAGAGC	rs12159555	chr22:20073323-20073323	+	C/G	seed
113	hsa-mir-3689b MI0016411	TGTGATATCATGGTTCCTGGGA	rs116838571	chr9:137742067-137742067	+	A/T	seed
114	hsa-mir-3622a MI0016013	CAGGCACGGGAGCTCAGGTGAG TCACCTGACCTCCCATGCCTGT	rs66683138	chr8:27559214-27559214	+	A/G	
			rs13276615	chr8:27559261-27559261	+	A/C	
115	hsa-mir-4274 MI0015884	CAGCAGTCCCTCCCCCTG	rs10655902	chr4:7461827-7461826	+	-/A/C/CCA	
			rs78759170	chr4:7461828-7461827	+	-/CAC	
			rs10655902	chr4:7461827-7461826	+	-/A/C/CCA	
			rs75530384	chr4:7461828-7461828	+	C/T	
			rs66511565	chr4:7461829-7461828	+	-/AC/CAC	
116	hsa-mir-3622b MI0016014	AGGCATGGGAGGTCAGGTGA TCACCTGAGCTCCCGTGCCTG	rs66683138	chr8:27559214-27559214	+	A/G	
			rs13276615	chr8:27559261-27559261	+	A/C	
117	hsa-mir-3678 MI0016079	TCCGTACAAACTCTGCTGTG CTGCAGAGTTTGTACGGACCGG	rs34571046	chr17:73402156-73402156	-	C/T	
118	hsa-mir-3679 MI0016080	TGAGGATATGGCAGGGAAGGGGA CTTCCCCCCAGTAATCTTCATC	rs79705373	chr2:134884717-134884717	+	A/G	
119	hsa-mir-3909 MI0016413	TGTCCTCTAGGGCCTGCAGTCT	rs34874675	chr22:35731712-35731712	+	-/G	
120	hsa-mir-3910-1 MI0016414	AAAAGGCATAAAACCAAGACA	rs76084273	chr9:94398600-94398600	+	A/C	
121	hsa-mir-3912 MI0016416	TAACGCATAATATGGACATGT	rs34887287	chr5:170813685-170813684	+	-/A	
122	hsa-mir-3910-2 MI0016431	AAAAGGCATAAAACCAAGACA	rs76084273	chr9:94398600-94398600	+	A/C	seed
123	hsa-mir-3924 MI0016432	ATATGTATATGTGACTGCTACT	rs77767763	chr10:59064260-59064260	+	C/G	
124	hsa-mir-3927 MI0016435	AGGTAGATATTTGATAGGCAT	rs80350479	chr9:112273768-112273768	+	A/C	
125	hsa-mir-3939 MI0016596	TACGCGCAGACCACAGGATGTC	rs76608449	chr6:167411333-167411333	+	C/G	seed
			rs75823810	chr6:167411334-167411334	+	C/T	seed
			rs73024232	chr6:167411337-167411337	+	A/G	seed

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele	REGION
126	hsa-mir-3928 MI0016438	GGAGGAACCTTGGAGCTTCGGC	rs60197191	chr22:31556051-31556050	+	-/AAAGCTC T TTGAAAA GCTGCC	
			rs72358191	chr22:31556059-31556058	+	-/TTTGAAA A GCTGCCG AAGCTC	
127	hsa-mir-642b MI0016685	AGACACATTTGGAGAGGGACCC	rs78902025	chr19:46178206-46178206	+	G/T	

Table A.2: Homology search (BLAT) of 298 potential *Homo sapiens* SNPs in presursor miRNA (pre-miRNA) and mature miRNAs.

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
1	hsa-mir-16-1 MI0000070	AGTATTAACTGTGCTGCTGA	rs72631826	chr13:50623143-50623143	-	C/T
2	hsa-mir-18a MI0000072	TAAGGTGCATCTAGTGCAGATAG	rs41275866	chr13:92003009-92003009	+	C/G
3	hsa-mir-25 MI0000082	AGGCGGAGACTTGGGCAATTG	rs41274221	chr7:99691200-99691200	+	C/T
4	hsa-mir-27a MI0000085	AGGGCTTAGCTGCTTGTGAGCA	rs895819	chr19:13947292-13947292	+	A/C/G/T
			rs11671784	chr19:13947296-13947296	+	A/G
5	hsa-mir-28 MI0000086	AAGGAGCTCACAGTCTATTGAG	rs78547906	chr3:188406636-188406636	+	C/T
6	hsa-mir-33a MI0000091	GTGCATTGTAGTTGCATTGCA	rs71764233	chr22:42296506-42298976	+	LARGE DELETION
			rs77809319	chr22:42296995-42296995	+	A/G
7	hsa-mir-93 MI0000095	AAAGTGCTGTTCGTGCAGGTAG	rs72631824	chr7:99691429-99691429	-	C/T
8	hsa-mir-96 MI0000098	TTTGGCACTAGCACATTTTTGCT	rs73159662	chr7:129414568-129414568	+	A/G
			rs41274239	chr7:129414574-129414574	+	A/G
9	hsa-mir-30c-2 MI0000254	CTGGGAGAAGGCTGTTTACTCT	rs113749278	chr6:72086720-72086720	+	A/G
10	hsa-mir-7-2 MI0000264	CAACAAATCCCAGTCTACCTAA	rs41276930	chr15:89155073-89155073	+	C/T
			rs75737367	chr15:89155084-89155084	+	A/C
11	hsa-mir-7-3 MI0000265	TGGAAGACTAGTGATTTTGTTGT	rs76199191	chr19:4770697-4770697	+	G/T
12	hsa-mir-10a MI0000266	TACCCTGTAGATCCGAATTTGTG	rs72631828	chr17:46657289-46657289	-	A/G
13	hsa-mir-34a MI0000268	TGGCAGTGTCTTAGCTGGTTGT	rs72631823	chr1:9211782-9211782	-	A/G
14	hsa-mir-182 MI0000272	TTTGGCAATGGTAGAACTCACACT	rs76481776	chr7:129410227-129410227	+	C/T
			rs77586312	chr7:129410228-129410228	+	A/G
			rs75953509	chr7:129410235-129410235	+	C/T
			rs80041074	chr7:129410239-129410239	+	C/T
15	hsa-mir-183 MI0000273	TATGGCACTGGTAGAATTCACT	rs72631833	chr7:129414804-129414804	-	G/T
16	hsa-mir-204 MI0000284	TTCCCTTTGTCATCCTATGCCT	rs112971695	chr9:73424988-73424988	+	A/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
17	hsa-mir-187 MI0000274	TCGTGTCTTGTGTTGCAGCCGG	rs114964240	chr18:33484783-33484783	+	C/T
			rs41274312	chr18:33484792-33484792	+	A/G
18	hsa-mir-199b MI0000282	CCCAGTGTTTAGACTATCTGTTC ACAGTAGTCTGCACATTGGTTA	rs72631835	chr9:131007109-131007109	-	C/T
19	hsa-mir-205 MI0000285	TCCTTCATTCCACCGGAGTCTG	rs113859371	chr1:209605546-209605546	+	C/T
20	hsa-mir-211 MI0000287	TTCCCTTTGTCATCCTTCGCCT	rs34520022	chr15:31357246-31357245	+	-/G
21	hsa-mir-215 MI0000291	ATGACCTATGAATTGACAGAC	rs72631834	chr1:220291292-220291292	-	C/T
22	hsa-mir-216a MI0000292	TAATCTCAGCTGGCAACTGTGA	rs41291179	chr2:56216090-56216090	+	A/T
23	hsa-mir-217 MI0000293	TACTGCATCAGGAACTGATTGGA	rs41291173	chr2:56210140-56210140	+	A/G
24	hsa-mir-222 MI0000299	CTCAGTAGCCAGTGTAGATCCT AGCTACATCTGGCTACTGGGT	rs72631825	chrX:45606471-45606471	-	A/G
25	hsa-mir-223 MI0000300	CGTGTATTTGACAAGCTGAGTT TGTCAGTTTGTCAAATACCCCA	rs34952329	chrX:65238768-65238767	+	-/C
26	hsa-mir-200b MI0000342	CATCTTACTGGGCAGCATTGGA TAATACTGCCTGGTAATGATGA	rs72563729	chr1:1102563-1102563	+	A/G
27	hsa-mir-30b MI0000441	TGTAAACATCCTACACTCAGCT CTGGGAGGTGGATGTTTACTTC	rs111424617	chr8:135812836-135812836	-	C/T
28	hsa-mir-124-2 MI0000444	CGTGTTCACAGCGGACCTTGAT TAAGGCACGCGGTGAATGCC	rs72631829	chr8:65291808-65291808	+	A/G
29	hsa-mir-128-1 MI0000447	TCACAGTGAACCGGTCTCTTT	rs117812383	chr2:136422988-136422988	+	A/G
30	hsa-mir-140 MI0000456	CAGTGGTTTTACCCTATGGTAG TACCACAGGGTAGAACCACGG	rs7205289	chr16:68524506-68524506	+	A/C
31	hsa-mir-141 MI0000457	CATCTTCCAGTACAGTGTTGGA TAACACTGTCTGGTAAAGATGG	rs34385807	chr12:6943606-6943605	+	-/C
32	hsa-mir-152 MI0000462	TCAGTGCATGACAGAACTTGG	rs67214557	chr17:43468372-43473043	+	LARGE DELETION
33	hsa-mir-9-2 MI0000467	TCTTTGGTTATCTAGCTGTATGA ATAAAGCTAGATAACCGAAAGT	rs41265488	chr5:87998503-87998503	+	A/T
34	hsa-mir-149 MI0000478	TCTGGCTCCGTGTCTTCACTCCC	rs71428439	chr2:241044173-241044173	+	A/G
		AGGGAGGGACGGGGGCTGTGC	rs2292832	chr2:241044176-241044176	+	C/T
35	hsa-mir-184 MI0000481	TGGACGGAGAACTGATAAGGGT	rs41280052	chr15:77289223-77289223	+	G/T
36	hsa-mir-193a MI0000487	TGGGTCTTTGCGGGCGAGATGA AACTGGCCTACAAAGTCCCAGT	rs60406007	chr17:26911146-26911146	+	G/T

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
37	hsa-mir-1-1 MI0000651	TGGAATGTAAAGAAGTATGTAT	rs6122014	chr20:60561960-60561960	-	C/T
38	hsa-mir-194-2 MI0000732	CCAGTGGGGCTGCTGTTATCTG	rs11231898	chr11:64415412-64415412	+	A/G
39	hsa-mir-106b MI0000734	TAAAGTGCTGACAGTGCAGAT CCGCACTGTGGGTACTTGCTGCTC	rs72631827	chr7:99529588-99529588	-	G/T
40	hsa-mir-299 MI0000744	TGGTTTACCGTCCCACAT TATGTGGGATGGTAAACCGCTT	rs72172875	chr14:100558894-100568278	+	LARGE DELETION
41	hsa-mir-130b MI0000748	ACTCTTTCCCTGTTGCACTAC CAGTGCAATGATGAAAGGGCAT	rs72631822	chr22:20337634-20337634	+	A/G
42	hsa-mir-365-2 MI0000769	AGGGACTTTCAGGGGCAGCTGTGT TAATGCCCCTAAAAATCCTTAT	rs35143473	chr17:29902519-29902519	+	-/T
43	hsa-mir-379 MI0000787	TGGTAGACTATGGAACGTAGG TATGTAACATGGTCCACTAACT	rs115827677	chr14:101488430-101488430	+	A/G
44	hsa-mir-326 MI0000808	CCTCTGGGCCCTTCCTCCAG	rs72561778	chr11:75046227-75046227	-	A/G
45	hsa-mir-339 MI0000815	TCCCTGTCCTCCAGGAGCTCACG	rs13232101	chr7:1062574-1062574	+	G/T
		TGAGCGCCTCGACGACAGAGCCG	rs72631831	chr7:1062656-1062656	-	A/G
46	hsa-mir-133b MI0000822	TTGGTCCCCTTCAACCAGCTA	rs112599381	chr6:52013754-52013754	+	C/T
47	hsa-mir-325 MI0000824	CCTAGTAGGTGTCCAGTAAGTGT	rs72631830	chrX:76225915-76225915	-	G/T
48	hsa-mir-345 MI0000825	CTGACTCCTAGTCCAGGGCTC	rs72631832	chr14:100774203-100774203	+	C/T
			rs114693307	chr14:100774289-100774289	+	A/C
49	hsa-mir-423 MI0001445	TGAGGGGCAGAGAGCGAGACTTT AGCTCGGTCTGAGGCCCCTCAGT	rs6505162	chr17:28444183-28444183	+	A/C
50	hsa-mir-449a MI0001648	TGGCAGTGTATTGTTAGCTGGT	rs112310158	chr5:54466378-54466378	+	A/G
51	hsa-mir-431 MI0001721	TGTCTTGCAGGCCGTCATGCA CAGGTCGTCTTGCAGGGCTTCT	rs76090066	chr14:101347355-101347355	+	C/T
52	hsa-mir-329-1 MI0001725	AACACACCTGGTTAACCTCTTT	rs34557733	chr14:101493130-101493129	+	-/A
53	hsa-mir-412 MI0002464	ACTTCACCTGGTCCACTAGCCGT	rs115831106	chr14:101531806-101531806	+	C/T
54	hsa-mir-484 MI0002468	TCAGGCTCAGTCCCCTCCCGAT	rs112103086	chr16:15737217-15737217	+	G/T
			rs112906136	chr16:15737218-15737218	+	A/G
55	hsa-mir-486 MI0002470	TCCTGTACTGAGCTGCCCCGAG	rs59908561	chr8:41517960-41517959	+	-/G
		CGGGGCAGCTCAGTACAGGAT	rs72177614	chr8:41517961-41517960	+	-/G
56	hsa-mir-489 MI0003124	GTGACATCACATATACGGCAGC	rs35930643	chr7:93113324-93113323	+	-/A
57	hsa-mir-496 MI0003136	TGAGTATTACATGGCCAATCT	rs79307187	chr14:101526957-101526957	+	A/C

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
58	hsa-mir-146b MI0003129	TGAGAACTGAATTCCATAGGCT TGCCCTGTGGACTCAGTTCTGG	rs76149940	chr10:104196269-104196269	+	C/T
			rs117717575	chr10:104196300-104196300	+	G/T
59	hsa-mir-202 MI0003130	TTCCTATGCATATACTTCTTTG AGAGGTATAGGGCATGGGAA	rs12355840	chr10:135061112-135061112	+	C/T
60	hsa-mir-492 MI0003131	AGGACCTGCGGGACAAGATTCTT	rs75413415	chr12:95228179-95228179	+	A/C
			rs2289030	chr12:95228286-95228286	-	C/G
61	hsa-mir-520f MI0003146	AAGTGCTTCCTTTTAGAGGGTT	rs75598818	chr19:54185492-54185492	+	A/G
62	hsa-mir-525 MI0003152	CTCCAGAGGGATGCACTTTCT GAAGGCGCTTCCCTTTAGAGCG	rs80268200	chr19:54200830-54200830	+	A/G
63	hsa-mir-521-2 MI0003163	AACGCACTTCCCTTTAGAGTGT	rs13382089	chr19:54219854-54219854	+	G/T
64	hsa-mir-520g MI0003166	ACAAAGTGCTTCCCTTTAGAGTGT	rs112328520	chr19:54225501-54225501	+	C/T
65	hsa-mir-516b-2 MI0003167	ATCTGGAGGTAAGAAGCACTTT TGCTTCCTTTCAGAGGGT	rs10583889	chr19:54228774-54228774	+	-/TT
			rs114381285	chr19:54228775-54228775	+	C/T
66	hsa-mir-526a-2 MI0003168	CTCTAGAGGGAAGCACTTTCTGT	rs113349469	chr19:54230219-54230219	+	A/T
67	hsa-mir-518a-1 MI0003170	ACAAAGTGCTTCCCTTTAGAGTGT	rs79646188	chr19:54225501-54225501	+	C/T
68	hsa-mir-516b-2 MI0003167	ATCTGGAGGTAAGAAGCACTTT TGCTTCCTTTCAGAGGGT	rs10583889	chr19:54228774-54228774	+	-/TT
			rs114381285	chr19:54228775-54228775	+	C/T
69	hsa-mir-526a-2 MI0003168	CTCTAGAGGGAAGCACTTTCTGT	rs113349469	chr19:54230219-54230219	+	A/T
70	hsa-mir-518a-1 MI0003170	CTGCAAAGGGAAGCCCTTTC	rs79646188	chr19:54234340-54234340	+	G/T
71	hsa-mir-518d MI0003171	CTCTAGAGGGAAGCACTTTCTG CAAAGCGCTTCCCTTTGGAGC	rs116220629	chr19:54238182-54238182	+	C/T
			rs74704964	chr19:54238208-54238208	+	C/T
72	hsa-mir-516b-1 MI0003172	ATCTGGAGGTAAGAAGCACTTT TGCTTCCTTTCAGAGGGT	rs79235448	chr19:54240136-54240136	+	C/G
73	hsa-mir-517c MI0003174	ATCGTGCATCCTTTTAGAGTGT	rs114627055	chr19:54244647-54244647	+	C/T
74	hsa-mir-520h MI0003175	ACAAAGTGCTTCCCTTTAGAGT	rs56013413	chr19:54245788-54245788	+	A/G
75	hsa-mir-521-1 MI0003176	AACGCACTTCCCTTTAGAGTGT	rs2561251	chr19:54251937-54251937	+	A/G
76	hsa-mir-516a-1 MI0003180	TTCTCGAGGAAAGAAGCACTTTC TGCTTCCTTTCAGAGGGT	rs2569389	chr19:54260002-54260002	-	A/G
77	hsa-mir-516a-2 MI0003181	TTCTCGAGGAAAGAAGCACTTTC TGCTTCCTTTCAGAGGGT	rs115975528	chr19:54264394-54264394	+	C/T
78	hsa-mir-499 MI0003183	TTAAGACTTGCAGTGATGTTT AACATCACAGCAAGTCTGTGCT	rs7267163	chr20:33578276-33578276	+	C/T

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
79	hsa-mir-513a-2 MI0003192	TTCACAGGGAGGTGTCAT TAAATTTCACCTTTCTGAGAAGG	rs111970838	chrX:146307449-146307449	+	G/T
80	hsa-mir-514-1 MI0003198	ATTGACACTTCTGTGAGTAGA	rs113816728	chrX:146360845-146360845	+	C/T
81	hsa-mir-532 MI0003205	CATGCCTTGAGTGTAGGACCGT	rs456615	chrX:49767832-49767832	+	A/G
		CCTCCCACACCCAAGGCTTGCA	rs456617	chrX:49767835-49767835	+	A/G
82	hsa-mir-553 MI0003558	AAAACGGTGAGATTTTGTTTT	rs112891767	chr1:100746855-100746855	+	C/T
83	hsa-mir-554 MI0003559	GCTAGTCCTGACTCAGCCAGT	rs79661940	chr1:151518284-151518284	+	G/T
84	hsa-mir-555 MI0003561	AGGGTAAGCTGAACCTCTGAT	rs113306711	chr1:155316214-155316214	+	C/T
85	hsa-mir-558 MI0003564	TGAGCTGCTGTACCAAAAT	rs72089144	chr2:32757231-32757230	+	-/GTGT
			rs71953654	chr2:32757232-32757231	+	-/TGTGTGTGTG
			rs35999329	chr2:32757239-32757238	+	-/TGTG
			rs72215615	chr2:32757240-32757239	+	-/TGTG
86	hsa-mir-559 MI0003565	TAAAGTAAATATGCACCAAAA	rs114803590	chr2:47604856-47604856	+	C/T
			rs58450758	chr2:47604866-47604866	+	C/T
87	hsa-mir-561 MI0003567	CAAAGTTTAAGATCCTTGAAGT	rs112456539	chr2:189162257-189162257	+	A/G
88	hsa-mir-564 MI0003570	AGGCAACATGGCCGAGAGGC	rs114636202	chr3:44903385-44903385	+	A/G
			rs2292181	chr3:44903434-44903434	+	C/G
89	hsa-mir-567 MI0003573	AGTATGTTCTTCCAGGACAGAAC	rs115707679	chr3:111831722-111831722	+	A/C
			rs76778672	chr3:111831736-111831736	+	-/AA
			rs67780840	chr3:111831725-111831724	+	-/A
			rs71704738	chr3:111831738-111831737	+	-/AAAA
90	hsa-mir-569 MI0003576	AGTTAATGAATCCTGGAAAGT	rs73037390	chr3:170824453-170824453	+	A/C
			rs116867000	chr3:170824459-170824459	+	A/G
91	hsa-mir-570 MI0003577	CGAAAACAGCAATTACCTTTGC	rs9860655	chr3:195426305-195426305	+	C/T
92	hsa-mir-581 MI0003588	TCTTGTGTTCTCTAGATCAGT	rs788517	chr5:53247386-53247386	-	A/G
93	hsa-mir-573 MI0003580	CTGAAGTGATGTGTAACTGATCAG	rs76014664	chr4:24521902-24521902	+	A/G
			rs113070835	chr4:24521822-24521822	+	C/T
			rs77543406	chr4:24521910-24521910	+	A/T
			rs78830737	chr4:24521904-24521904	+	A/G
94	hsa-mir-576 MI0003583	ATTCTAATTTCTCCACGTCTTT AAGATGTGGAAAAATTGGAATC	rs77639117	chr4:110409933-110409933	+	A/T
95	hsa-mir-583 MI0003590	CAAAGAGGAAGGTCCCATTAC	rs10697860	chr5:95414852-95414851	+	-/ATAAA

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
96	hsa-mir-577 MI0003584	TAGATAAAATATTGGTACCTG	rs79560193	chr4:115577921-115577921	+	A/G
			rs34115976	chr4:115577997-115577997	+	C/G
97	hsa-mir-580 MI0003587	TTGAGAATGATGAATCATTAGG	rs115089112	chr5:36148057-36148057	+	C/T
			rs73080005	chr5:36148054-36148054	+	G/T
98	hsa-mir-585 MI0003592	TGGGCGTATCTGTATGCTA	rs115473017	chr5:168690611-168690611	+	C/T
			rs62376934	chr5:168690612-168690612	+	A/G
99	hsa-mir-548a-1 MI0003593	CAAAACTGGCAATTACTTTTGC	rs12197631	chr6:18572056-18572056	+	G/T
100	hsa-mir-586 MI0003594	TATGCATTGTATTTTTAGGTCC	rs73735310	chr6:45165473-45165473	+	A/G
101	hsa-mir-550a-1 MI0003600	AGTGCCTGAGGGAGTAAGAGCCC TGTCTTACTCCCTCAGGCACAT	rs71528599	chr7:30329467-30329467	+	C/T
102	hsa-mir-550a-2 MI0003601	AGTGCCTGAGGGAGTAAGAGCCC TGTCTTACTCCCTCAGGCACAT	rs113464681	chr7:32772637-32772639	+	-/TGT
103	hsa-mir-595 MI0003607	GAAGTGTGCCGTGGTGTGTCT	rs117344178	chr7:158325411-158325411	+	C/T
			rs114151270	chr7:158325415-158325415	+	C/T
			rs4909237	chr7:158325503-158325503	+	C/T
104	hsa-mir-596 MI0003608	AAGCCTGCCCGGCTCCTCGGG	rs116450906	chr8:1765409-1765409	+	C/T
			rs111606748	chr8:1765435-1765435	+	C/T
105	hsa-mir-597 MI0003609	TGTGTGTCACTCGATGACCACTGT	rs79397096	chr8:9599276-9599276	+	A/G
106	hsa-mir-548a-3 MI0003612	AAAAGTAATTGCGAGTTTTACC	rs71297276	chr8:105496638-105496638	+	-/TGAAAGTAAT
107	hsa-mir-602 MI0003615	GACACGGGCGACAGCTGCGGCCC	rs116636751	chr9:140732963-140732963	+	C/T
108	hsa-mir-603 MI0003616	CACACACTGCAATTACTTTTGCT	rs79500031	chr10:24564632-24564632	+	G/T
			rs11014002	chr10:24564653-24564653	+	C/T
109	hsa-mir-604 MI0003617	AGGCTGCGGAATTCAGGAC	rs2368393	chr10:29833998-29833998	-	C/T
			rs2368392	chr10:29834003-29834003	-	C/T
110	hsa-mir-605 MI0003618	TAAATCCCATGGTGCCTTCTCCT	rs2043556	chr10:53059406-53059406	-	A/G
111	hsa-mir-607 MI0003620	GTTCAAATCCAGATCTATAAC	rs115264283	chr10:98588440-98588440	+	A/G
			rs12778876	chr10:98588495-98588495	+	A/T
			rs12780546	chr10:98588496-98588496	+	A/T
112	hsa-mir-609 MI0003622	AGGGTGTTTCTCTCATCTCT	rs74154754	chr10:105978581-105978581	+	C/G
113	hsa-mir-611 MI0003624	GCGAGGACCCCTCGGGGTCTGAC	rs111585800	chr11:61560026-61560026	+	A/T
114	hsa-mir-612 MI0003625	GCTGGGCAGGGCTTCTGAGCTCCTT	rs550894	chr11:65211940-65211940	-	G/T
			rs12803915	chr11:65211979-65211979	+	A/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
115	hsa-mir-548c MI0003630	AAAAGTAATTGCGGTTTTTGCC CAAAAATCTCAATTACTTTTGC	rs17120527	chr12:65016300-65016300	+	A/G
116	hsa-mir-617 MI0003631	AGACTTCCCATTTGAAGGTGGC	rs12815353	chr12:81226326-81226326	+	C/G
			rs113739044	chr12:81226358-81226358	+	A/G
117	hsa-mir-618 MI0003632	AAACTCTACTTGTCCTTCTGAGT	rs2682818	chr12:81329536-81329536	+	A/C
118	hsa-mir-619 MI0003633	GACCTGGACATGTTTGTGCCCAGT	rs34651680	chr12:109230733-109230732	+	-/G
119	hsa-mir-631 MI0003645	AGACCTGGCCCAGACCTCAGC	rs5745925	chr15:75645967-75645967	+	-/CT
120	hsa-mir-633 MI0003648	CTAATAGTATCTACCACAATAAA	rs75781970	chr17:61021604-61021604	+	G/T
			rs17759989	chr17:61021611-61021611	+	A/G
121	hsa-mir-635 MI0003650	ACTTGGGCACTGAAACAATGTCC	rs77279010	chr17:66420592-66420592	+	A/G
122	hsa-mir-639 MI0003654	ATCGCTGCGGTTGCGAGCGCTGT	rs45556632	chr19:14640403-14640403	+	C/G
			rs35149836	chr19:14640439-14640439	+	A/G
123	hsa-mir-645 MI0003660	TCTAGGCTGGTACTGCTGA	rs35645123	chr20:49202355-49202354	+	-/A
124	hsa-mir-646 MI0003661	AAGCAGCTGCCTCTGAGGC	rs6513496	chr20:58883534-58883534	+	C/T
125	hsa-mir-647 MI0003662	GTGGCTGCACTCACTTCCTTC	rs73147065	chr20:62574006-62574006	+	A/G
126	hsa-mir-650 MI0003665	AGGAGGCAGCGCTCTCAGGAC	rs5996397	chr22:23165340-23165340	+	C/G
127	hsa-mir-662 MI0003670	TCCCACGTTGTGGCCCAGCAG	rs74656628	chr16:820215-820215	+	A/T
128	hsa-mir-656 MI0003678	AATATTATACAGTCAACCTCT	rs58834075	chr14:101533093-101533093	+	C/T
129	hsa-mir-758 MI0003757	TTTGTGACCTGGTCCACTAACC	rs72172875	chr14:101489141-101498525	+	LARGE DELETION
130	hsa-mir-620 MI0003634	ATGGAGATAGATATAGAAAT	rs77703604	chr12:116586414-116586414	+	A/C
			rs10549054	chr12:116586415-116586416	+	-/TA
			rs5801168	chr12:116586426-116586427	+	-/AT
			rs3043743	chr12:116586435-116586436	+	-/T/TA
			rs34551929	chr12:116586436-116586436	+	-/A
			rs34380284	chr12:116586436-116586437	+	-/C
131	hsa-mir-663 MI0003672	AGGCGGGGCGCCGCGGGACCGC	rs28670321	chr20:26188824-26188824	+	C/T
			rs2019798	chr20:26188846-26188846	-	G/T
			rs7266947	chr20:26188912-26188912	+	A/C
132	hsa-mir-1323 MI0003786	TCAAAACTGAGGGGCATTTTCT	rs78952474	chr19:54175287-54175287	+	G/T
133	hsa-mir-1185-2 MI0003821	AGAGGATACCCTTTGTATGTT	rs11844707	chr14:101510613-101510613	+	A/G
134	hsa-mir-449c MI0003823	TAGGCAGTGTATTGCTAGCGGCTGT TTGCTAGTTGCACTCCTCTCTGT	rs75661995	chr5:54468166-54468166	+	C/T

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
135	hsa-mir-1283-1 MI0003832	TCTACAAAGGAAAGCGCTTTCT	rs57111412	chr19:54191743-54191743	+	A/G
136	hsa-mir-759 MI0004065	GCAGAGTGCAAACAATTTTGAC	rs77079459	chr13:53384256-53384256	+	G/T
137	hsa-mir-891a MI0005524	TGCAACGAACCTGAGCCACTGA	rs5965990	chrX:145109356-145109356	+	A/G
138	hsa-mir-300 MI0005525	TATACAAGGGCAGACTCTCTCT	rs12894467	chr14:101507727-101507727	+	C/T
139	hsa-mir-888 MI0005537	TACTCAAAAAGCTGTCAGTCA GACTGACACCTCTTTGGGTGAA	rs5965660	chrX:145076302-145076302	+	G/T
140	hsa-mir-147b MI0005544	GTGTGCGGAAATGCTTCTGCTA	rs56073218	chr15:45725255-45725255	+	C/G
141	hsa-mir-543 MI0005565	AAACATTCGCGGTGCACTTCTT	rs72172875	chr14:101489141-101498525	+	LARGE DELETION
142	hsa-mir-920 MI0005712	GGGGAGCTGTGGAAGCAGTA	rs66686007	chr12:24365358-24365358	+	-/GTTGT
143	hsa-mir-934 MI0005756	TGTCTACTACTGGAGACACTGG	rs73558572	chrX:135633045-135633045	+	A/G
144	hsa-mir-941-1	CACCCGGCTGTGTGCACATGTGC	rs4809383	chr20:62550780-62550780	+	C/T
			rs7262125	chr20:62550791-62550791	+	A/G
			rs56202554	chr20:62550794-62550794	+	C/T
			rs7268785	chr20:62550806-62550806	+	C/G
			rs55795631	chr20:62550877-62550877	+	C/T
145	hsa-mir-944 MI0005769	AAATTATTGTACATCGGATGAG	rs75715827	chr3:189547735-189547735	+	C/T
146	hsa-mir-941-1	CACCCGGCTGTGTGCACATGTGC	rs6089780	chr20:62550880-62550880	+	A/G
			rs6090019	chr20:62550890-62550890	+	C/T
147	hsa-mir-941-2	CACCCGGCTGTGTGCACATGTGC	rs55795631	chr20:62550877-62550877	+	C/T
			rs6089780	chr20:62550880-62550880	+	A/G
			rs6090019	chr20:62550890-62550890	+	C/T
			rs72366056	chr20:62551075-62551101	+	-/AGGACAGC GC CACGGAAGAGGACGCAC
			rs34604519	chr20:62551169-62551169	+	C/G
			rs7360929	chr20:62551199-62551199	+	C/T
148	hsa-mir-943	CTGACTGTTGCCGTCCTCCAG	rs3034718	chr4:1988189-1988188	+	-/CT
			rs35401110	chr4:1988190-1988189	+	-/CT
			rs1077020	chr4:1988193-1988193	+	C/T
149	hsa-mir-941-4	CACCCGGCTGTGTGCACATGTGC	rs7360929	chr20:62551199-62551199	+	C/T
			rs12625445	chr20:62551225-62551225	+	C/G
			rs35544770	chr20:62551272-62551272	+	A/G
			rs12625454	chr20:62551281-62551281	+	C/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
150	hsa-mir-297 MI0005775	ATGTATGTGTGCATGTGCATG	rs113258805	chr4:111781746-111781746	+	A/G
151	hsa-mir-1181 MI0006274	CCGTCGCCGCCACCCGAGCCG	rs2569788	chr19:10514159-10514159	+	C/G
152	hsa-mir-1178 MI0006271	TTGCTCACTGTTCTTCCCTAG	rs7311975	chr12:120151493-120151493	+	C/T
			rs74614893	chr12:120151501-120151501	+	A/G
153	hsa-mir-1227 MI0006316	CGTGCCACCCTTTTCCCCAG	rs74807305	chr19:2234086-2234086	+	A/T
154	hsa-mir-1229 MI0006319	CTCTCACCACTGCCCTCCCACAG	rs2291418	chr5:179225324-179225324	+	C/T
155a	hsa-mir-1234 MI0006324	TCGGCCTGACCACCCACCCCAC	rs75169642	chr8:145625535-145625535	+	C/G
			rs2291134	chr8:145625536-145625536	+	C/G
155b	hsa-mir-1234 MI0006324	TCGGCCTGACCACCCACCCCAC	rs66769762	chr8:145625537-145625537	+	-/GG
			rs76908615	chr8:145625542-145625542	+	-/CC
156	hsa-mir-1206 MI0006339	TGTTCATGTAGATGTTTAAGC	rs2114358	chr8:129021179-129021179	-	C/T
157	hsa-mir-1286 MI0006348	TGCAGGACCAAGATGAGCCCT	rs71312743	chr22:20236701-20236701	+	A/C
158	hsa-mir-1287 MI0006349	TGCTGGATCAGTGGTTCGAGTC	rs78666598	chr10:100155019-100155019	+	A/T
159	hsa-mir-663b MI0006336	GGTGGCCCGGCCGTGCCTGAGG	rs62165009	chr2:133014579-133014579	+	A/G
			rs75245503	chr2:133014602-133014602	+	C/G
			rs74853538	chr2:133014612-133014612	+	A/C
			rs78327226	chr2:133014619-133014619	+	C/T
			rs78867621	chr2:133014640-133014640	+	C/T
160	hsa-mir-1208 MI0006341	TCACTGTTCAGACAGGCGGA	rs56863230	chr8:129162366-129162366	+	C/G
			rs2648841	chr8:129162433-129162433	-	A/C
			rs4822739	chr22:26951185-26951185	+	C/G
			rs12161068	chr22:26951215-26951215	+	C/T
161	hsa-mir-1289-2	TGGAGTCCAGGAATCTGCATTTTT	rs35296450	chr5:132763295-132763295	+	C/G
			rs35731356	chr5:132763305-132763304	+	-/G
			rs74634410	chr5:132763305-132763305	+	G/T
			rs75310044	chr5:132763335-132763335	+	A/C
162	hsa-mir-1290 MI0006352	TGGATTTTTGGATCAGGGA	rs75705742	chr1:19223639-19223639	+	A/G
163	hsa-mir-1294 MI0006356	TGTGAGGTTGGCATTGTTGTCT	rs13186787	chr5:153726769-153726769	+	A/G
164	hsa-mir-1299 MI0006359	TTCTGGAATCCTACGTGAGGGA	rs62555121	chr9:69002271-69002271	+	A/T
165	hsa-mir-548l MI0006361	AAAAGTATTTGCGGGTTTTGTC	rs11020790	chr11:94199710-94199710	+	C/T
166	hsa-mir-1302-1 MI0006362	TTAAATTTGAATTTCAAGTAAA	rs74647838	chr12:113132901-113132901	+	A/G
167	hsa-mir-548f-5 MI0006378	AAAAACTGTAATTACTTTT	rs60180387	chrX:32659592-32659592	+	C/T

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
168a	hsa-mir-1303 MI0006370	TTTAGAGACGGGGTCTTGCTCT	rs77055126	chr5:154065348-154065348	+	C/T
			rs75378399	chr5:154065368-154065368	+	A/T
			rs75538180	chr5:154065383-154065383	+	A/T
168b	hsa-mir-1303 MI0006370	TTTAGAGACGGGGTCTTGCTCT	rs34889453	chr5:154065383-154065383	+	-/A
			rs33982250	chr5:154065385-154065385	+	-/A
169	hsa-mir-1304 MI0006371	TTTGAGGCTACAGTGAGATGTG	rs2155248	chr11:93466866-93466866	-	A/C
			rs79462725	chr11:93466919-93466919	+	C/G
170	hsa-mir-1248 MI0006383	ACCTTCTTGTATAAGCACTGTGCTAAA	rs73063489	chr3:186504532-186504532	+	C/T
171	hsa-mir-1255a MI0006389	AGGATGAGCAAAGAAAGTAGATT	rs28664200	chr4:102251501-102251501	+	C/T
172	hsa-mir-1253 MI0006387	AGAGAAGAAGATCAGCCTGCA	rs7217038	chr17:2651377-2651377	+	A/T
			rs77498226	chr17:2651405-2651405	+	G/T
173	hsa-mir-1260 MI0006394	ATCCCACCTCTGCCACCA	rs28909969	chr14:77732628-77732628	-	-/T
174	hsa-mir-1265 MI0006401	CAGGATGTGGTCAAGTGTTGTT	rs11259096	chr10:14478618-14478618	+	C/T
175	hsa-mir-548o MI0006402	CCAAAACTGCAGTTACTTTTGC	rs79628069	chr7:102046296-102046296	+	G/T
176	hsa-mir-1267 MI0006404	CCTGTTGAAGTGTAATCCCCA	rs76080861	chr13:108183538-108183538	+	C/T
177	hsa-mir-1274a MI0006410	GTCCCTGTTCAGGCGCCA	rs318039	chr5:41475766-41475766	+	C/T
178	hsa-mir-548h-4 MI0006414	AAAAGTAATCGCGGTTTTTGTC	rs73235382	chr8:26906437-26906437	+	A/G/T
			rs74273836	chr8:26906437-26906441	+	-/AGTAA
179	hsa-mir-1275 MI0006415	GTGGGGGAGAGGCTGTC	rs76156362	chr6:33967787-33967787	+	A/T
180	hsa-mir-548i-1 MI0006421	AAAAGTAATTGCGGATTTTGCC	rs34864809	chr3:125509375-125509374	+	-/G
181	hsa-mir-548i-4 MI0006424	AAAAGTAATTGCGGATTTTGCC	rs72632467	chrX:83480764-83480764	+	A/G
182	hsa-mir-1282 MI0006429	TCGTTTGCCTTTTTCTGCTT	rs11269	chr15:44085909-44085909	+	G/T
183	hsa-mir-1283-2 MI0006430	TCTACAAAGGAAAGCGCTTTCT	rs71363366	chr19:54261549-54261549	+	C/G
184	hsa-mir-1292 MI0006433	TGGGAACGGGTTCCGGCAGACGCT	rs73576045	chr20:2633466-2633466	+	C/T
185	hsa-mir-1255b-2 MI0006436	CGGATGAGCAAAGAAAGTGGTT	rs79639536	chr1:167967958-167967958	+	A/G
186	hsa-mir-1307 MI0006444	ACTCGGCGTGGCGTCGGTCGTG	rs7911488	chr10:105154089-105154089	+	A/G
187	hsa-mir-1322 MI0006653	GATGATGCTGCTGATGCTG	rs76657610	chr8:10682923-10682923	+	C/T
188	hsa-mir-1324 MI0006657	CCAGACAGAATTCTATGCACTTTC	rs75174508	chr3:75680010-75680010	+	C/T
189	hsa-mir-320d-1 MI0008190	AAAAGCTGGGTTGAGAGGA	rs74826059	chr13:41301987-41301987	+	A/G
190	hsa-mir-1908 MI0008329	CGGCGGGGACGGCGATTGGTC	rs174561	chr11:61582708-61582708	+	C/T
191	hsa-mir-1911 MI0008332	TGAGTACCGCCATGTCTGTTGGG	rs79553157	chrX:113997746-113997746	+	A/G
192	hsa-mir-2276 MI0011282	TCTGCAAGTGTCAGAGGCGAGG	rs74399078	chr13:24736638-24736638	+	C/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
193	hsa-mir-2110 MI0010629	TTGGGGAAACGGCCGCTGAGTG	rs75407574	chr10:115933876-115933876	+	A/G
			rs17091403	chr10:115933905-115933905	+	C/T
194	hsa-mir-2116 MI0010635	GTTCTTAGCATAGGAGGTCT CCTCCCATGCCAAGAACTCCC	rs3838847	chr15:59463451-59463451	-	-/A
195	hsa-mir-2117 MI0010636	TGTTCTCTTTGCCAAGGACAG	rs7207008	chr17:41522213-41522213	+	A/T
			rs76511826	chr17:41522221-41522221	+	A/G
196	hsa-mir-2278 MI0011285	GAGAGCAGTGTGTGTTGCCTGG	rs356125	chr9:97572244-97572244	+	A/G
197	hsa-mir-3118-3 MI0014133	TGTGACTGCATTATGAAAATTCT	rs9440733	chr1:143424215-143424215	+	G/T
198	hsa-mir-3119-1 MI0014134	TGGCTTTTAACTTTGATGGC	rs58602811	chr1:170120575-170120575	+	G/T
199	hsa-mir-3119-2 MI0014135	TGGCTTTTAACTTTGATGGC	rs58602811	chr1:170120575-170120575	+	G/T
200	hsa-mir-3123 MI0014139	CAGAGAATTGTTTAATC	rs78240175	chr1:241295617-241295617	+	A/G
201	hsa-mir-3125 MI0014142	TAGAGGAAGCTGTGGAGAGA	rs72536414	chr2:12877501-12877500	+	-/A
			rs78852835	chr2:12877501-12877501	+	A/G
			rs5829384	chr2:12877502-12877501	+	-/A
202	hsa-mir-3131 MI0014151	TCGAGGACTGGTGGAAGGGCCTT	rs71974827	chr2:219923412-219923411	+	-/G
			rs57408770	chr2:219923419-219923418	+	-/AAG
203	hsa-mir-3132 MI0014152	TGGGTAGAGAAGGAGCTCAGAGGA	rs12996671	chr2:220413812-220413812	+	A/T
			rs12997198	chr2:220413813-220413813	+	C/G
204	hsa-mir-3135 MI0014156	TGCCTAGGCTGAGACTGCAGTG	rs6787734	chr3:20179097-20179097	+	C/T
205	hsa-mir-544b MI0014159	ACCTGAGGTTGTGCATTTCTAA	rs10934682	chr3:124451312-124451312	+	G/T
206	hsa-mir-548t MI0014164	CAAAAGTGATCGTGGTTTTTG	rs73872515	chr4:174189360-174189360	+	A/C
207	hsa-mir-3141 MI0014165	GAGGGCGGGTGGAGGAGGA	rs936581	chr5:153975576-153975576	+	A/G
208	hsa-mir-3143 MI0014167	ATAACATTGTAAAGCGCTTCTTTCG	rs17737028	chr6:27115467-27115467	+	A/G
209	hsa-mir-548u MI0014168	CAAAGACTGCAATTACTTTTGCGC	rs4994089	chr6:57254939-57254939	+	G/T
			rs5876554	chr6:57254951-57254950	+	-/T
			rs33917213	chr6:57254952-57254951	+	-/T
			rs67187462	chr6:57254953-57254952	+	-/T
			rs2894842	chr6:57254955-57254955	+	A/G
210	hsa-mir-3144 MI0014169	AGGGGACCAAAGAGATATATAG ATATACCTGTTCGGTCTCTTTA	rs68035463	chr6:120336327-120336327	+	A/C
211	hsa-mir-3151 MI0014178	GTGGGGCAATGGGATCAGGT	rs35605502	chr8:104166903-104166902	+	-/C
			rs72544670	chr8:104166905-104166904	+	-/C

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
212	hsa-mir-3161 MI0014191	CTGATAAGAACAGAGGCCCAGAT	rs73466882	chr11:48118374-48118374	+	A/T
213	hsa-mir-3166 MI0014196	GCAGACAATGCCTACTGGCCTA	rs35854553	chr11:87909673-87909673	-	A/T
214	hsa-mir-1260b MI0014197	ATCCCACCACTGCCACCAT	rs34913856	chr11:96074649-96074649	+	-/G
215	hsa-mir-3167 MI0014198	AGGATTTCAGAAATACTGGTGT	rs670637	chr11:126858392-126858392	-	C/T
			rs634171	chr11:126858406-126858406	-	A/T
216	hsa-mir-3170 MI0014201	CTGGGGTTCTGAGACAGACAGT	rs76289985	chr13:98860827-98860827	+	C/G
217	hsa-mir-3171 MI0014202	AGATGTATGGAATCTGTATATATC	rs35170395	chr14:28102477-28102478	+	-/TA
			rs67062245	chr14:28102484-28102485	+	-/AT
218	hsa-mir-323b MI0014206	AGGTTGTCCGTGGTGAGTTCGCA CCCAATACACGGTCGACCTCTT	rs56103835	chr14:101522556-101522556	+	C/T
219	hsa-mir-3175 MI0014209	CGGGGAGAGAACGCAGTGACGT	rs1439619	chr15:93447631-93447631	-	A/C
220	hsa-mir-3176 MI0014210	ACTGGCCTGGGACTACCGG	rs8054514	chr16:593277-593277	+	G/T
221	hsa-mir-3177 MI0014211	TGCACGGCACTGGGGACACGT	rs28575325	chr16:1785038-1785038	+	A/G
222	hsa-mir-3183 MI0014225	GCCTCTCTCGGAGTCGCTCGGA	rs72812091	chr17:925742-925742	+	A/G
			rs2663345	chr17:925764-925764	-	C/T
223	hsa-mir-3188 MI0014232	AGAGGCTTTGTGCGGATACGGGG	rs7247237	chr19:18392894-18392894	+	C/T
			rs7247767	chr19:18392913-18392913	+	A/G
			rs41510649	chr19:18392936-18392936	-	A/G
224	hsa-mir-3189 MI0014233	CCCCTTGGGTCTGATGGGGTAG	rs71779635	chr19:18497275-18499093	+	LARGE DELETION
225	hsa-mir-320e MI0014234	AAAGCTGGGTTGAGAAGG	rs10423365	chr19:47212593-47212593	+	A/G
226	hsa-mir-3192 MI0014237	TCTGGGAGGTTGTAGCAGTGGAA	rs6111978	chr20:18451300-18451300	+	C/T
			rs11907020	chr20:18451325-18451325	+	C/T
227	hsa-mir-3196 MI0014241	CGGGGCGGCAGGGGCCTC	rs7363975	chr20:61870162-61870162	+	A/C
228	hsa-mir-3156-3 MI0014242	AAAGATCTGGAAGTGGGAGACA	rs2747232	chr21:14778721-14778721	-	A/T
229	hsa-mir-3199-1 MI0014247	AGGGACTGCCTTAGGAGAAAGTT	rs78805657	chr22:28316591-28316591	+	G/T
			rs80166589	chr22:28316592-28316591	+	-/G
			rs75321888	chr22:28316593-28316592	+	-/G
230	hsa-mir-4293 MI0015826	CAGCCTGACAGGAACAG	rs12780876	chr10:14425204-14425204	+	A/T
231	hsa-mir-4294 MI0015827	GGGAGTCTACAGCAGGG	rs12776349	chr10:50193625-50193625	+	A/G
232	hsa-mir-4298 MI0015830	CTGGGACAGGAGGAGGAGGCAG	rs75966923	chr11:1880730-1880730	+	A/C
233	hsa-mir-4305 MI0015835	CCTAGACACCTCCAGTTC	rs67976778	chr13:40238175-40238175	+	C/T
			rs4636784	chr13:40238258-40238258	+	C/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
234	hsa-mir-4309 MI0015837	CTGGAGTCTAGGATTCCA	rs78860350	chr14:103006026-103006026	+	A/T
			rs12879262	chr14:103006047-103006047	+	C/G
235	hsa-mir-4308 MI0015839	TCCCTGGAGTTTCTTCTT	rs28477407	chr14:55344901-55344901	+	C/T
236	hsa-mir-4312 MI0015842	GGCCTTGTTCCTGTCCCCA	rs79711957	chr15:69094259-69094259	+	A/C
237	hsa-mir-4318 MI0015847	CACTGTGGGTACATGCT	rs11873400	chr18:35237129-35237129	+	A/G
238	hsa-mir-4320 MI0015849	GGGATTCTGTAGCTTCCT	rs4430847	chr18:47652913-47652913	-	A/G
239	hsa-mir-4317 MI0015850	ACATTGCCAGGGAGTTT	rs74513330	chr18:6374394-6374394	+	A/G
240	hsa-mir-4324 MI0015854	CCCTGAGACCCTAACCTTAA	rs79465964	chr19:49812123-49812123	+	C/T
241	hsa-mir-4259 MI0015858	CAGTTGGGTCTAGGGGTCAGGA	rs35180578	chr1:159869829-159869828	+	-/C
242	hsa-mir-4260 MI0015859	CTTGGGGCATGGAGTCCCA	rs7544976	chr1:209796810-209796810	+	G/T
243	hsa-mir-4253 MI0015860	AGGGCATGTCCAGGGGGT	rs66652227	chr1:23189707-23189707	+	-/G
244	hsa-mir-4326 MI0015866	TGTTCCTCTGTCTCCCAGAC	rs6062431	chr20:61918164-61918164	+	C/G
245	hsa-mir-4267 MI0015871	TCCAGCTCGGTGGCAC	rs55940978	chr2:110827551-110827551	+	C/T
			rs59793833	chr2:110827558-110827558	+	A/G
246	hsa-mir-4268 MI0015874	GGCTCCTCCTCTCAGGATGTG	rs4674470	chr2:220771223-220771223	+	C/T
247	hsa-mir-4270 MI0015878	TCAGGGAGTCAGGGGAGGGC	rs79922312	chr3:15537776-15537776	+	A/G
248	hsa-mir-4274 MI0015884	CAGCAGTCCCTCCCCCTG	rs12512664	chr4:7461769-7461769	+	A/G
249	hsa-mir-4281 MI0015885	GGGTCCCGGGGAGGGGGG	rs34983866	chr5:176056492-176056492	-	-/C
250	hsa-mir-4277 MI0015886	GCAGTTCTGAGCACAGTACAC	rs12523324	chr5:1708983-1708983	+	A/G
251	hsa-mir-4279 MI0015887	CTCTCCTCCCGGCTTC	rs34157017	chr5:31936264-31936263	+	-/A
252	hsa-mir-4285 MI0015891	GCGGCGAGTCCGACTCAT	rs34606939	chr7:101936373-101936372	+	-/G
			rs2960269	chr7:101936374-101936374	-	C/G
			rs2906714	chr7:101936375-101936375	+	-/C/T
			rs1293864	chr7:101936397-101936397	-	G/T
			rs35647990	chr7:101936420-101936419	+	-/C
			rs1293863	chr7:101936422-101936422	-	-/A/G
			rs1293862	chr7:101936435-101936435	-	A/T
253	hsa-mir-4286 MI0015894	ACCCCACTCCTGGTACC	rs80245767	chr8:10524531-10524531	+	A/C
254	hsa-mir-4328 MI0015904	CCAGTTTTCCCAGGATT	rs35376760	chrX:78156694-78156693	+	-/G
255	hsa-mir-3605 MI0015995	TGAGGATGGATAGCAAGGAAGCC CCTCCGTGTTACCTGTCCTCTAG	rs80176156	chr1:33798031-33798031	+	C/G
256	hsa-mir-3609 MI0015999	CAAAGTGATGAGTAATACTGGCTG	rs34109026	chr7:98479274-98479274	+	-/T

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
257	hsa-mir-3612 MI0016002	AGGAGGCATCTTGAGAAATGGA	rs1683709	chr12:128778703-128778703	-	C/T
258	hsa-mir-3615 MI0016005	TCTCTCGGCTCCTCGCGGCTC	rs745666	chr17:72744798-72744798	-	C/G
259	hsa-mir-3617 MI0016007	AAAGACATAGTTGCAAGATGGG	rs80142005	chr20:44333749-44333749	+	A/G
260	hsa-mir-3619 MI0016009	TCAGCAGGCAGGCTGGTGCAGC	rs73888764	chr22:46486996-46486996	+	A/G
261	hsa-mir-3620 MI0016011	TCACCCTGCATCCCGCACCCAG	rs2070960	chr1:228284991-228284991	+	C/T
262	hsa-mir-3647 MI0016047	CTGAAGTGATGATTCACATTCAT AGAAAATTTTTGTGTGTCTGATC	rs28429778	chr16:70563410-70563410	+	A/C
263	hsa-mir-3659 MI0016060	TGAGTGTTGTCTACGAGGGCA	rs553122	chr1:38554942-38554942	-	A/G
264	hsa-mir-3652 MI0016052	CGGCTGGAGGTGTGAGGA	rs62747560	chr12:104324231-104324231	+	-/GGGGTGG
			rs71440517	chr12:104324238-104324244	+	-/GGGGTGG
			rs17797090	chr12:104324266-104324266	+	A/G
265	hsa-mir-3657 MI0016057	TGTGTCCCATTATTGGTGATT	rs77419179	chr12:112475403-112475403	+	C/T
			rs71083196	chr12:112475428-112475427	+	-/C
			rs71083197	chr12:112475430-112475429	+	-/G
			rs71719442	chr12:112472575-112476739	+	LARGE DELETION
266	hsa-mir-3660 MI0016061	ACTGACAGGAGAGCATTTTGA	rs34630557	chr5:89312452-89312451	+	-/C
			rs75102645	chr5:89312487-89312487	+	A/G
267	hsa-mir-3664 MI0016065	AACTCTGTCTTCACTCATGAGT	rs75728268	chr11:70718466-70718466	+	G/T
268	hsa-mir-3667 MI0016068	AAAGACCCATTGAGGAGAAGGT ACCTTCCTCTCCATGGGTCTTT	rs76749295	chr22:49937076-49937076	+	A/G
269	hsa-mir-3671 MI0016072	ATCAAATAAGGACTAGTCTGCA	rs521188	chr1:65523519-65523519	+	A/G
270	hsa-mir-3679 MI0016080	TGAGGATATGGCAGGGAAGGGGA CTTCCCCCCAGTAATCTTCATC	rs10175383	chr2:134884697-134884697	+	C/G
			rs6430498	chr2:134884700-134884700	+	A/G
271a	hsa-mir-3673 MI0016074	ATGGAATGTATATACGGAATA	rs57824231	chr8:130508130-130508130	+	-/AT
			rs55921050	chr8:130508142-130508142	+	C/T
			rs60861880	chr8:130508149-130508149	+	C/T
271b	hsa-mir-3673 MI0016074	ATGGAATGTATATACGGAATA	rs60525947	chr8:130508165-130508165	+	G/T
			rs58699099	chr8:130508172-130508172	+	C/T
			rs58872489	chr8:130508179-130508178	+	-/CA
272	hsa-mir-3674 MI0016075	ATTGTAGAACCTAAGATTGGCC	rs75404472	chr8:1749333-1749333	+	C/T
273	hsa-mir-3682 MI0016083	TGATGATACAGGTGGAGGTAG	rs1047873	chr2:54076332-54076332	-	A/G
274	hsa-mir-3686 MI0016087	ATCTGTAAGAGAAAGTAAATGA	rs6997249	chr8:130496365-130496365	+	A/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
275	hsa-mir-3683 MI0016084	TGCGACATTGGAAGTAGTATCA	rs6943868	chr7:7106600-7106600	+	A/T
			rs6977967	chr7:7106636-7106636	+	A/G
276	hsa-mir-3714 MI0016135	GAAGGCAGCAGTGCTCCCCTGT	rs55835744	chr3:16974725-16974725	+	C/T
			rs75653184	chr3:16974739-16974739	+	A/C
277	hsa-mir-3180-4 MI0016408	TGGGGCGGAGCTTCCGGAG	rs5016496	chr16:15248707-15248707	-	C/G
			rs5016497	chr16:15248717-15248717	-	C/G
			rs75000738	chr16:15248720-15248720	+	A/C
278	hsa-mir-3180-5 MI0016409	TGGGGCGGAGCTTCCGGAG	rs2855372	chr16:2186054-2186054	-	G/T
			rs2021041	chr16:2186129-2186129	-	C/T
279	hsa-mir-3689b MI0016411	TGTGATATCATGGTTCCTGGGA	rs74405398	chr9:137741987-137741987	+	A/G
			rs71506933	chr9:137741989-137741989	+	C/G
			rs34933499	chr9:137741993-137741993	+	A/C/G
			rs76317538	chr9:137741994-137741994	+	A/G
			rs71483239	chr9:137742013-137742013	+	C/G
			rs76578598	chr9:137742017-137742017	+	A/G
			rs79438120	chr9:137742018-137742018	+	A/G
			rs35679521	chr9:137742041-137742041	+	A/G
			rs113626871	chr9:137742043-137742043	+	G/T
280	hsa-mir-3688 MI0016089	TATGGAAAGACTTTGCCACTCT	rs70962655	chr4:160050032-160050031	+	-/C
281	hsa-mir-3910-1 MI0016414	AAAAGGCATAAAACCAAGACA	rs12344333	chr9:94398543-94398543	+	C/T
			rs67339585	chr9:94398581-94398581	+	C/T
282	hsa-mir-3908 MI0016412	GAGCAATGTAGGTAGACTGTTT	rs74446573	chr12:124021052-124021052	+	G/T
			rs61953551	chr12:124021054-124021054	+	G/T
283	hsa-mir-3909 MI0016413	TGTCCTCTAGGGCCTGCAGTCT	rs9607265	chr22:35731697-35731697	+	A/G
284	hsa-mir-3911 MI0016415	TGTGTGGATCCTGGAGGAGGCA	rs75848623	chr9:130452968-130452968	+	G/T
285	hsa-mir-3922 MI0016429	TCTGGCCTTGACTTGACTCTTT	rs61938575	chr12:104985443-104985443	+	A/G
286	hsa-mir-3910-2 MI0016431	AAAAGGCATAAAACCAAGACA	rs67339585	chr9:94398581-94398581	+	C/T
287	hsa-mir-3928 MI0016438	GGAGGAACCTTGGAGCTTCGGC	rs5997893	chr22:31556103-31556103	+	A/G
288	hsa-mir-3936 MI0016592	TAAGGGGTGTATGGCAGATGCA	rs61745813	chr5:131701185-131701185	+	C/T
			rs367805	chr5:131701279-131701279	-	A/G
289	hsa-mir-550b-1 MI0016686	TCTTACTCCCTCAGGCACTG	rs71528599	chr7:30329467-30329467	+	C/T
290	hsa-mir-548z MI0016688	CAAAAACCGCAATTACTTTTGCA	rs17120527	chr12:65016300-65016300	+	A/G

	miRNA	Mature miRNA Sequence	SNP ID	Position	Strand	Allele
291	hsa-mir-3938 MI0016594	AATTCCCTTGTAGATAACCCGG	rs35071197	chr3:55886523-55886522	+	-/G
			rs59684995	chr3:55886574-55886574	+	A/T
			rs10575780	chr3:55886581-55886582	+	-/AA
			rs28647346	chr3:55886608-55886608	+	A/G
292	hsa-mir-3939 MI0016596	TACGCGCAGACCACAGGATGTC	rs80032204	chr6:167411298-167411298	+	C/T
			rs77840042	chr6:167411300-167411300	+	C/T
			rs75692943	chr6:167411301-167411301	+	A/G
			rs70979794	chr6:167411314-167411313	-	1552 BP INSERTION
			rs13216014	chr6:167411314-167411314	+	C/T
			rs12200860	chr6:167411344-167411344	+	C/T
			rs77072520	chr6:167411362-167411362	+	A/G
			rs73024234	chr6:167411388-167411388	+	C/G
293	hsa-mir-941-3 MI0005765	CACCCGGCTGTGTGCACATGTGC	rs6089780	chr20:62550880-62550880	+	A/G
			rs6090019	chr20:62550890-62550890	+	C/T
			rs55795631	chr20:62550877-62550877	+	C/T
294	hsa-mir-3689a MI0016090	TGTGATATCATGGTTCCTGGGA CTGGGAGGTGTGATATCGTGGT	rs71501303	chr9:137741197-137741803	+	CCACGAT/GCACGGG
295	hsa-mir-3171 MI0014202	AGATGTATGGAATCTGTATATATC	rs61972532	chr14:28102427-28102427	+	A/G

Table A.3: Relative Quantification (RQ) of mature miRNA-125a was calculated by applying to the $2^{-\Delta\Delta CT}$ analyses.

Sample	Wild Type Probe	Mutant Probe	Endo miR-16	Endo RNU44	Average endogenous	Wild type ΔCT	Mutant ΔCT
Empty Vector (EV)	30.99	37.32	27.31	26.30	26.80	4.18	10.52
	30.58	36.36	27.28	25.40	26.34	4.24	10.03
	30.67	36.00	27.56	25.52	26.54	4.13	9.46
	30.84	36.11	27.34	26.55	26.95	3.89	9.17
	31.22	37.35	27.82	26.50	27.16	4.06	10.19
	31.41	37.23	27.59	27.00	27.29	4.11	9.93
	29.28	34.90	26.14	23.06	24.60	4.68	10.30
	29.34	34.20	26.27	23.29	24.78	4.56	9.42
	29.33	35.14	25.40	23.34	24.37	4.96	10.76
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Wild Type miR-125a	23.33		28.03	26.43	27.23	-3.90	
	23.32		27.34	26.17	26.75	-3.43	
	23.31		27.15	26.31	26.73	-3.42	
	23.18		27.14	26.23	26.68	-3.51	
	23.12		27.83	25.12	26.47	-3.35	
	23.15		27.59	25.59	26.59	-3.44	
	21.65		25.17	23.14	24.16	-2.51	
	21.74		26.10	23.53	24.82	-3.08	
	21.71		25.57	23.22	24.39	-2.68	
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Mutant miR-125aM		21.77	26.48	24.92	25.70		-3.93
		22.46	26.76	25.01	25.89		-3.42
		21.71	26.66	24.21	25.44		-3.73
		22.93	27.08	25.09	26.08		-3.15
		22.31	27.58	24.31	25.94		-3.63
		23.11	26.44	24.22	25.33		-2.22
		22.01	25.59	24.48	25.03		-3.03
		22.16	25.58	24.67	25.13		-2.97
		21.79	25.69	24.66	25.18		-3.39

Sample	Wild type ΔCT	Mutant ΔCT	ΔΔCT	Relative Quatification (RQ)	Average RQ	Log RQ	Average Log	SD	CV (%)
Empty Vector (EV)	4.18	10.52	0.00	1.00	1.00	0.00	0.00	0.00	0.00
	4.24	10.03	0.00	1.00					
	4.13	9.46	0.00	1.00					
	3.89	9.17	0.00	1.00					
	4.06	10.19	0.00	1.00					
	4.11	9.93	0.00	1.00					
	4.68	10.30	0.00	1.00					
	4.56	9.42	0.00	1.00					
	4.96	10.76	0.00	1.00					
Wild Type miR-125a	-3.90		-8.08	270.50	192.39	2.43	2.28	0.07	3.24
	-3.43		-7.67	203.59		2.31			
	-3.42		-7.55	187.53		2.27			
	-3.51		-7.40	168.90		2.23			
	-3.35		-7.41	169.84		2.23			
	-3.44		-7.55	187.01		2.27			
	-2.51		-7.19	145.61		2.16			
	-3.08		-7.64	198.78		2.30			
	-2.68		-7.64	199.74		2.30			
Mutant miR-125aM		-3.93	-14.45	22334.71	11197.50	4.35	3.99	0.25	6.23
		-3.42	-13.45	11186.72		4.05			
		-3.73	-13.18	9309.58		3.97			
		-3.15	-12.32	5102.54		3.71			
		-3.63	-13.82	14462.21		4.16			
		-2.22	-12.15	4546.37		3.66			
		-3.03	-13.32	10254.72		4.01			
		-2.97	-12.39	5363.65		3.73			
		-3.39	-14.15	18217.03		4.26			

$$P_{t\text{- test}} = 1.119 \times 10^{-12}$$

Key for Table A.3: [miR-16 and RNU44 = endogenous controls; ΔCT = delta CT (CT sample – CT endogenous control); ΔΔCT = delta delta CT (ΔCT wild-type miRNA-125a - ΔCT mutagenized miRNA-125a); RQ = relative quantification; CV = correlation variation; SD = standard deviation]

Table A.4: Relative Quantification (RQ) of mature miRNA-124-3 was calculated by applying to the $2^{-\Delta\Delta CT}$ analyses.

Sample	Wild Type Probe	Mutant Probe	Endo miR-16	Endo RNU44	Average endogenous	Wild type ΔCT	Mutant ΔCT
Empty Vector (EV)	28.48	32.14	20.15	21.80	20.97	7.50	11.17
	28.46	32.37	20.29	22.08	21.19	7.27	11.18
	28.92	32.26	20.36	22.06	21.21	7.71	11.05
	28.87	35.79	24.41	27.08	25.74	3.13	10.05
	29.01	36.67	24.56	27.15	25.86	3.15	10.82
	29.07	37.92	24.33	26.80	25.57	3.50	12.36
	28.84	38.46	23.41	24.09	23.75	5.09	14.71
	29.02	37.57	23.34	24.13	23.73	5.29	13.84
	29.23	38.84	23.92	24.24	24.08	5.15	14.76
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Wild Type miR-124	27.54		23.75	25.18	24.46	3.08	
	27.30		24.30	25.77	25.04	2.26	
	27.15		24.12	26.32	25.22	1.94	
	25.08		27.21	33.79	30.50	-5.43	
	25.14		26.71	33.37	30.04	-4.91	
	25.26		26.60	31.36	28.98	-3.72	
	26.63		25.48	27.42	26.45	0.17	
	26.45		25.65	28.18	26.92	-0.47	
	26.76		25.26	27.84	26.55	0.21	
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Mutant miR-124M		24.01	26.06	25.44	25.75		-1.74
		23.92	24.83	23.44	24.13		-0.21
		24.52	25.25	23.22	24.23		0.29
		24.28	23.12	29.00	26.06		-1.78
		24.48	22.97	28.23	25.60		-1.12
		24.68	23.61	26.62	25.12		-0.43
		24.69	25.21	27.53	26.37		-1.68
		24.86	25.28	27.95	26.61		-1.76
		24.64	25.51	27.76	26.63		-2.00

Sample	Wild type ΔCT	Mutant ΔCT	ΔΔCT	Relative Quatification (RQ)	Average RQ	Log RQ	Average Log	SD	CV (%)
Empty Vector (EV)	7.50	11.17	0.00	1.00	1.00	0.00	0.00	0.00	0.00
	7.27	11.18	0.00	1.00					
	7.71	11.05	0.00	1.00					
	3.13	10.05	0.00	1.00					
	3.15	10.82	0.00	1.00					
	3.50	12.36	0.00	1.00					
	5.09	14.71	0.00	1.00					
	5.29	13.84	0.00	1.00					
	5.15	14.76	0.00	1.00					
Wild Type miR-124	3.08		-4.42	21.45	112.83	1.33	1.83	0.45	24.75
	2.26		-5.01	32.31		1.51			
	1.94		-5.78	54.83		1.74			
	-5.43		-8.56	376.11		2.58			
	-4.91		-8.06	266.78		2.43			
	-3.72		-7.22	149.03		2.17			
	0.17		-4.92	30.26		1.48			
	-0.47		-5.76	54.06		1.73			
	0.21		-4.94	30.65		1.49			
Mutant miR-124M		-1.74	-12.91	7691.24	30330.34	3.89	4.03	0.68	16.97
		-0.21	-11.40	2693.94		3.43			
		0.29	-10.77	1743.78		3.24			
		-1.78	-11.83	3628.10		3.56			
		-1.12	-11.93	3914.20		3.59			
		-0.43	-12.79	7067.58		3.85			
		-1.68	-16.40	86295.64		4.94			
		-1.76	-15.60	49529.49		4.69			
		-2.00	-16.75	110409.13		5.04			

$$P_{t\text{- test}} = 5.163 \times 10^{-7}$$

Key for Table A.4: [miR-16 and RNU44 = endogenous controls; ΔCT = delta CT (CT sample – CT endogenous control); ΔΔCT = delta delta CT (ΔCT wild-type miRNA-124-3 - ΔCT variant miRNA-124-3); RQ = relative quantification; CV = correlation variation; SD = standard deviation]

Table A.5: Relative Quantification (RQ) of mature miRNA-662 was calculated by applying to the $2^{-\Delta\Delta CT}$ analyses.

Sample	Wild Type Probe	Mutant Probe	Endo miR-16	Endo RNU44	Average endogenous	Wild type ΔCT	Mutant ΔCT
Empty Vector (EV)	36.54	35.25	24.56	27.15	25.86	10.68	9.39
	35.69	35.98	24.33	26.80	25.57	10.13	10.41
	36.31	35.15	23.41	24.09	23.75	12.56	11.40
	35.88	34.41	23.34	24.13	23.73	12.15	10.68
	34.65	32.23	20.15	21.80	20.97	13.68	11.26
	35.52	31.04	20.29	22.08	21.19	14.34	9.85
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Wild Type miR-662	34.77		24.95	29.64	27.29	7.48	
	34.80		25.38	29.61	27.50	7.30	
	37.62		24.42	28.04	26.23	11.39	
	35.80		24.18	28.06	26.12	9.68	
	36.57		26.18	27.15	26.66	9.91	
	36.23		25.48	26.75	26.11	10.12	
Sample	**Wild Type Probe**	**Mutant Probe**	**Endo miR-16**	**Endo RNU44**	**Average endogenous**	**Wild type ΔCT**	**Mutant ΔCT**
Mutant miR-662M		34.18	28.11	32.89	30.50		3.68
		36.01	27.71	33.27	30.49		5.52
		31.88	25.30	28.06	26.68		5.20
		32.87	25.62	28.28	26.95		5.92
		31.29	24.20	27.33	25.77		5.52
		29.44	24.48	28.04	26.26		3.18

Sample	Wild type ΔCT	Mutant ΔCT	ΔΔCT	Relative Quatification (RQ)	Average RQ	Log RQ	Average Log	SD	CV (%)
Empty Vector (EV)	10.68	9.39	0.00	1.00	1.00	0.00	0.00	0.00	0.00
	10.13	10.41	0.00	1.00					
	12.56	11.40	0.00	1.00					
	12.15	10.68	0.00	1.00					
	13.68	11.26	0.00	1.00					
	14.34	9.85	0.00	1.00					
Wild Type miR-662	7.48		-3.21	9.23	9.40	0.97	0.89	0.32	36.38
	7.30		-2.83	7.09		0.85			
	11.39		-1.18	2.26		0.36			
	9.68		-2.46	5.52		0.74			
	9.91		-3.77	13.63		1.13			
	10.12		-4.22	18.66		1.27			
Mutant miR-662M		3.68	-5.71	52.35	56.30	1.72	1.70	0.22	13.05
		5.52	-4.89	29.65		1.47			
		5.20	-6.20	73.41		1.87			
		5.92	-4.76	27.01		1.43			
		5.52	-5.74	53.54		1.73			
		3.18	-6.67	101.83		2.01			

$$P_{t\text{- test}} = 4.551x\ 10^{-4}$$

Key for Table A.5: [miR-16 and RNU44 = endogenous controls; ΔCT = delta CT (CT sample – CT endogenous control); ΔΔCT = delta delta CT (ΔCT type wild miRNA-662 - ΔCT variant miRNA-662); RQ = relative quantification; CV = correlation variation; SD = standard deviation]

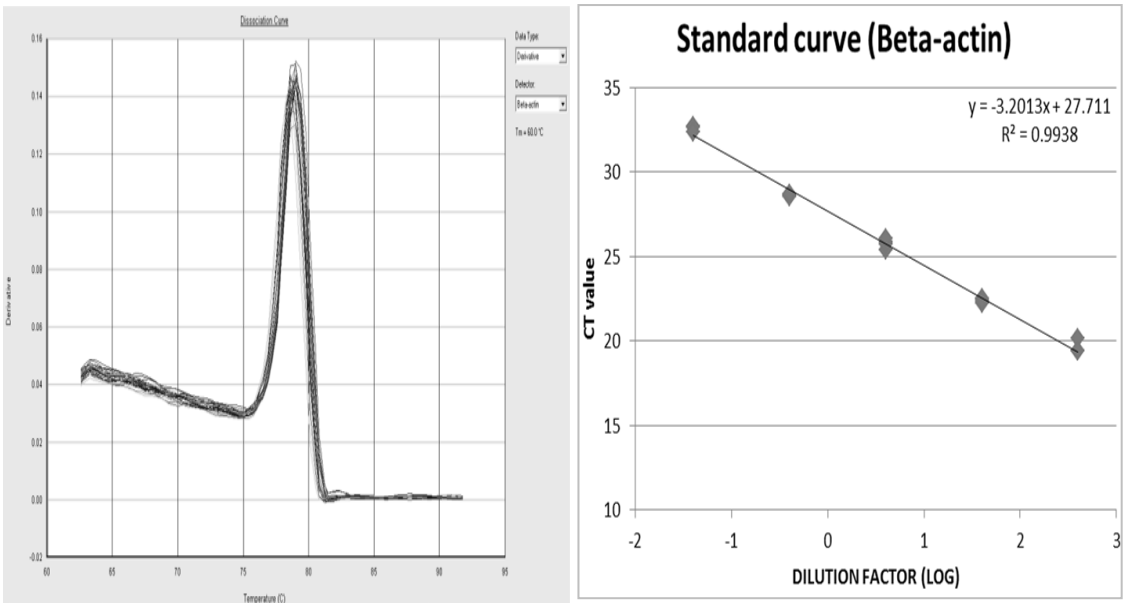

Figure A.1: Dissociation curve for Beta-actin primers showing one peak (above); standard curve for Beta-actin primers (below).

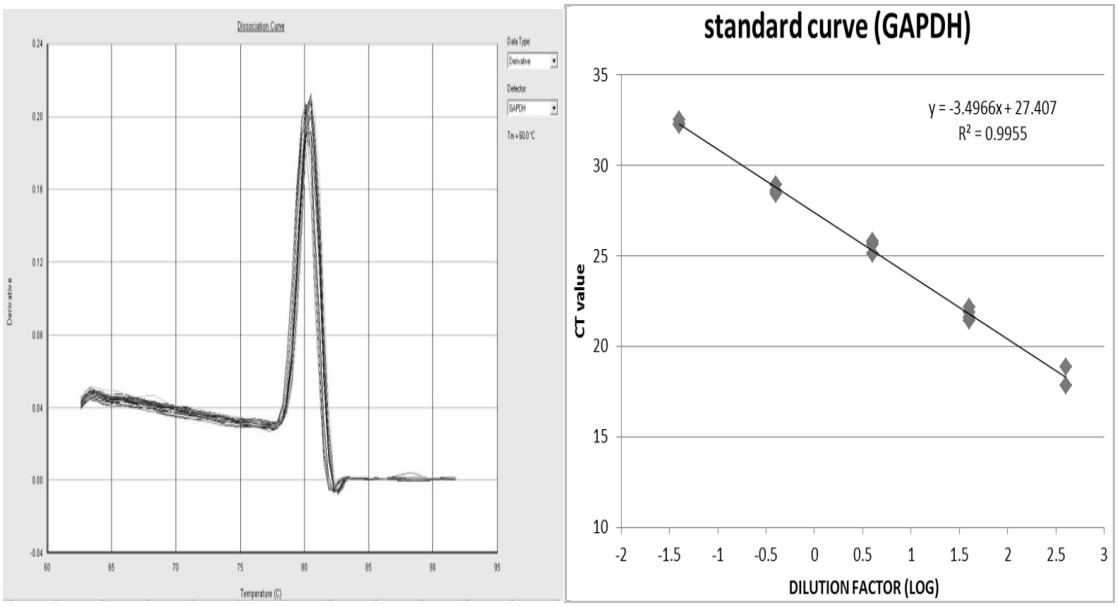

Figure A.2: Dissociation curve for GAPDH primers showing one peak (above); standard curve for GAPDH primers (below).

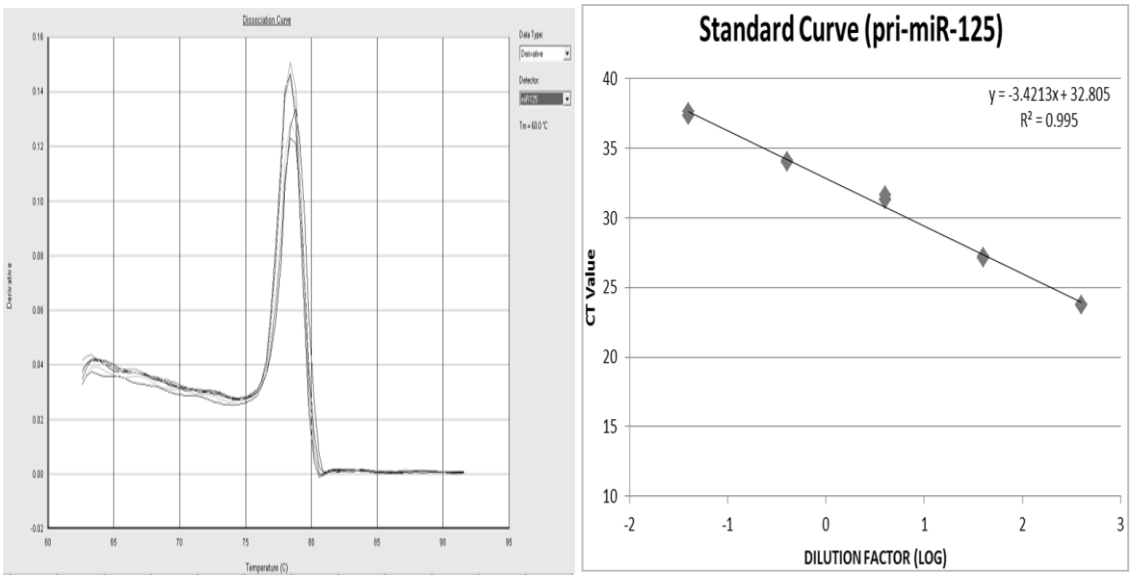

Figure A.3: Dissociation curve for primary miR-125a primers showing one peak (above); standard curve for primary miR-125a primers (below).

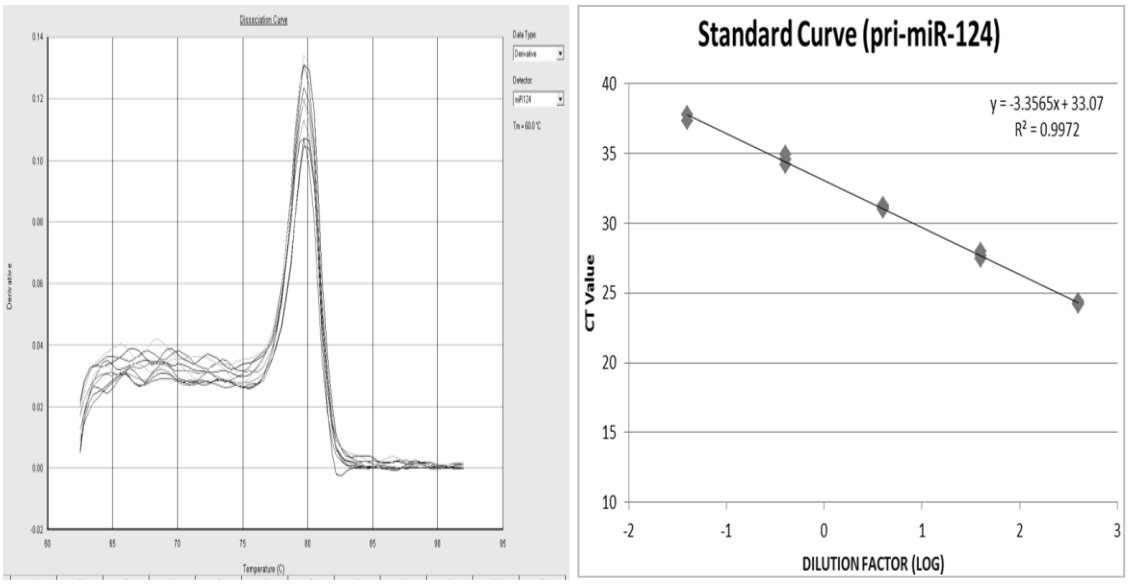

Figure A.4: Dissociation curve for pri-miR-124-3 primers showing one peak (above); standard curve for for pri-miR-124-3 primers (below).

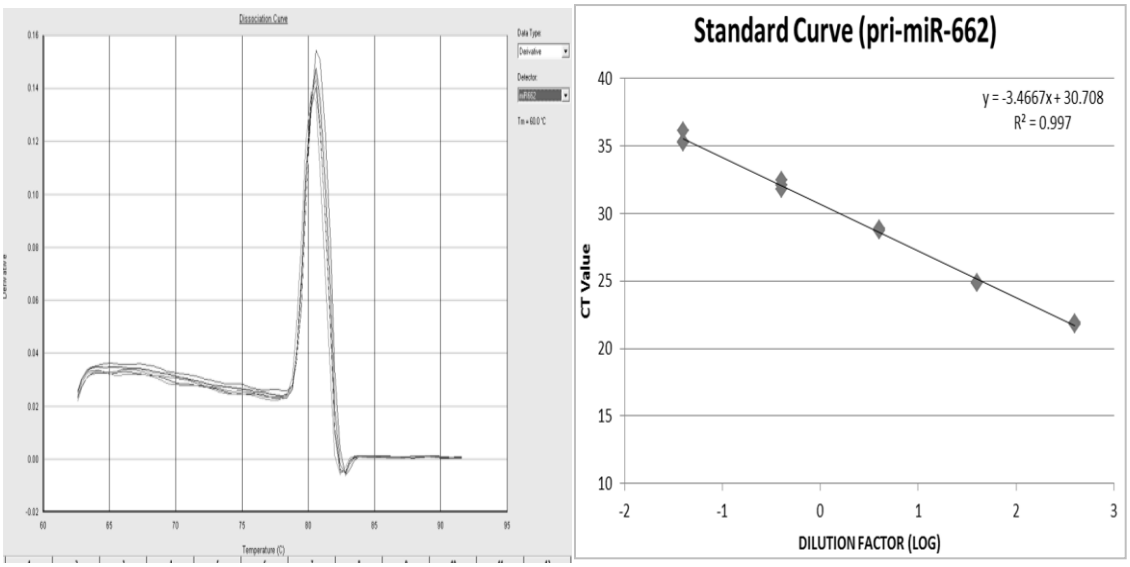

Figure A.5: Dissociation curve for pri-miR-662 primers showing one peak (above); standard curve for pri-miR-662 primers (below).

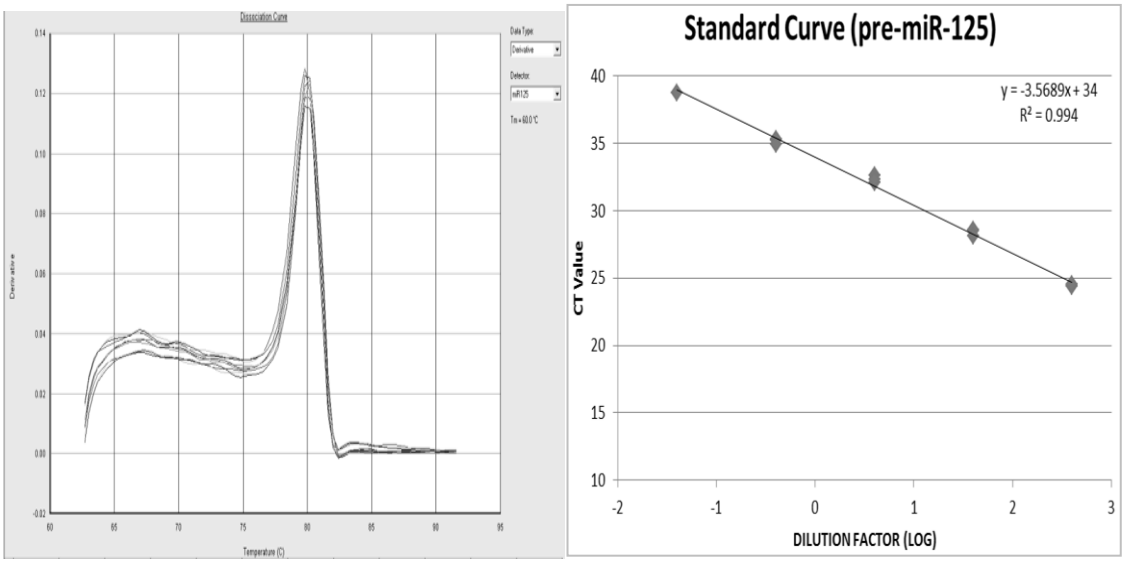

Figure A.6: Dissociation curve for precursor miR-125a primers showing one peak (above); standard vurce for precursor miR-125a primers (below).

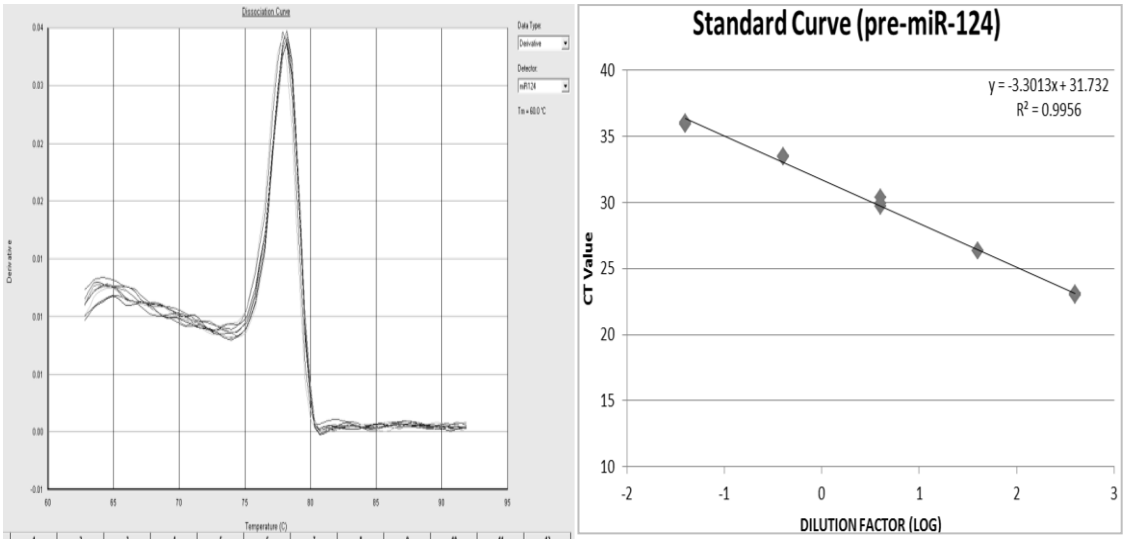

Figure A.7: Dissociation curve for pre-miR-124-3 primers showing one peak (above); standard curve for pre-miR-124-3 primers (below).

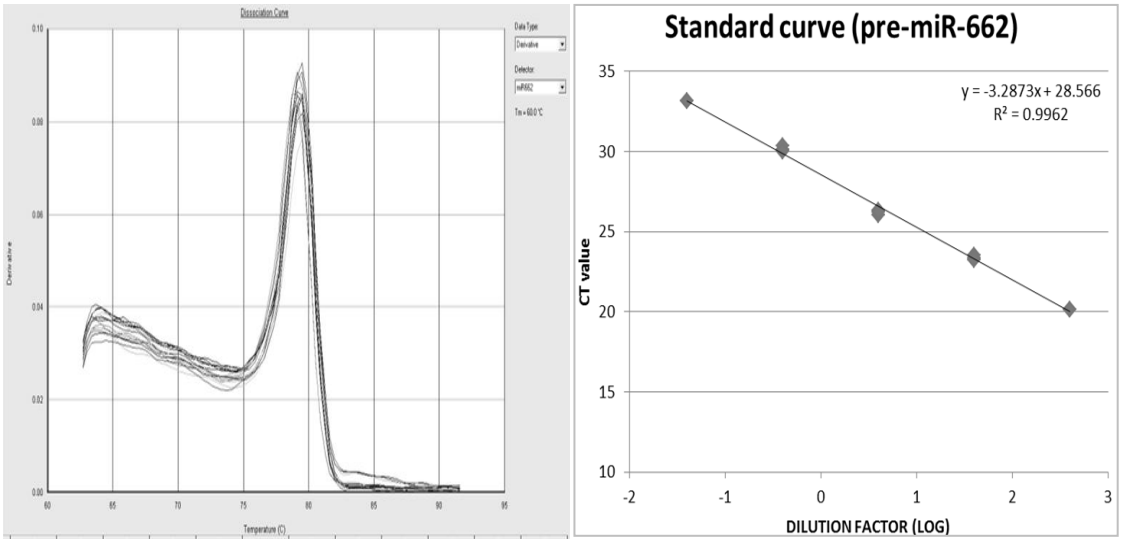

Figure A.8: Dissociation curve for pre-miR-662 primers showing one peak (above); standard curve for pre-miR-662 primers (below).