Dicer によるマイクロ RNA 前駆体（pre-miRNAs）切断反応に及ぼす小分子

A Study of The Effects of Small Molecules on Dicer-mediated Cleavage of Precursor miRNAs (pre-miRNAs)

Ph.D. Thesis

By

Muhammad Nurrohman Sidiq
Department of Chemistry
Graduate School of Science
Osaka University

Supervisor
Professor Kazuhiko Nakatani
Department of Chemistry
Graduate School of Science
Osaka University

Department of Regulatory Bioorganic Chemistry
SANKEN (The Institute of Scientific and Industrial Research)
Osaka University

ABSTRACT

MicroRNA (miRNA) is a small (18-22 nucleotides) and evolutionary conserved non-coding RNA that is expressed as a post-transcriptional regulator of living cells and is known to be implicated in various cellular processes and diseases. On the normal condition, miRNA was tightly regulated in the cells, however, the aberrant expression of this type of non-coding RNA would result in cancer, and this type of miRNA is commonly referred to as oncomirs. Inhibition is preferred to overcome this unfavorable overexpression.

A combination screening strategy was used to find a small molecule with the potential to be an inhibitor of miRNA production. This type of screening strategy was a hybrid of target-based screening and cell-based screening. The goal of this combination was to overcome the limitations of target-based screening, which did not take into account the physiological aspects of the living organism, and also cell-based screening, that considered as mechanism-agnostic screening. The combination of both types of screening would increase the relevance of screening results to physiological conditions and make it easier to track the mechanism of action behind the inhibition.

Uterine corpus endometrial carcinoma (UCEC) was chosen to apply those screening concepts due to the lack of alternative drugs available to cure this kind of cancer. Several UCEC-associated miRNAs reported by Favier A., et al. then were used to demonstrate the inhibition of small molecules to the production of UCEC-associated miRNAs (miR-182, miR-31, miR-30d). Nakatani group, on the other hand, developed small molecules with nucleobase recognition sites, such as guanine recognition by N-Acyl-2-amino-7-methyl-1,8-naphthyridine, adenine recognition by 7-methyl-2-oxo-1,8-naphthyridine (azaquinolone), and cytosine recognition by protonated 2-amino-7-methyl-1,8-naphthyridine. These small molecules are designed to specifically bind the complementary nucleobase on the nucleic acids. If those small molecules can bind to miRNA precursors, they may inhibit oncogenic miRNA production.

The first chapter of this dissertation described the framework and background of the research. In chapter 2, the use of Real-time PCR (qPCR) to determine the kinetical properties of an *in-vitro* Dicer reaction was investigated. However, due to a number of challenges, the results of this experiment did not meet the expected results.

In chapter 3, We demonstrated a multistep qPCR-based screening of an in-house chemical library that targets UCEC-associated miRNA production using the screening strategy that

combined the target-based screening and cell-based screening assay. *In-vitro* Dicer-mediated processing of pre-miR-182, pre-miR-31, and pre-miR-30d was used for the initial screening. The first screening yielded 48 different compounds with significant inhibition effects on miRNA production. The inhibitory effect of the identified compounds on the biogenesis of the miRNA targets on the cells was then investigated. We discovered eight hit compounds with potential inhibitory effects on pre-miR-182 and pre-miR-31 processing in vitro and in cells. Furthermore, the interaction of eight compounds with pre-miRNAs was studied using the Surface Plasmon Resonance (SPR) assay and gel analysis. Among eight hit compounds, only 2 compounds showed favorable binding to pre-miRNAs. This suggested two possible ways of inhibition may occur, first, the compounds bind to the pre-miRNA and interfere with the Dicer-mediated cleavage processing, or the compounds directly interact with Dicer and affected the cleavage processing.

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Abbreviation

AGO I	Argonaute RISC Component 1
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CCK-8	Cell-counting kit 8
cDNA	complementary DNA
CT value	Cycle Threshold Value
DANP	N,N'-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine
dNTP	deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration (agency within the United States Department of Health and Human Services)
IC_{50}	Inhibition Concentration- 50
Km	Michaelis Constant
MBLs	Mismatch Binding Ligands
miR-X	microRNA-X
miRNA	microRNA
MOA	Mechanism of Action
NA	Naphtiridine Azaquinolone
ND	Naphtiridine Dimer
PCR	Polymerase Chain Reaction
pre-miRNA	precursor microRNA
qPCR	quantitative / Real-time Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acids
RNA Pol II	RNA Polymerase II
RT	Reverse Transcription
RT-qPCR	Reverse Transcription - quantitative / Real-time Polymerase Chain Reaction
SA	Streptavidin
SPR	Surface Plasmon Resonance
TCGA	The Cancer Genome Atlas
TRBP	Transactivation Response element RNA-binding Protein
UCEC	Uterine Corpus Endometrial Carcinoma
Vmax	Maximum rate of reaction
WST	Water-soluble Tetrazolium

CHAPTER I
General Introduction

1.1 MicroRNA (miRNA) as a Post-transcriptional Gene Regulator and Cancer Therapeutics

miRNAs are a class of evolutionary conserved small non-coding RNA with 18-22 nucleotides in length, which regulate gene expression through translational repression and mRNA degradation. (1,2) The biogenesis of miRNAs as illustrated in Figure 1.1, begins with the transcription of the miRNA gene in the nucleus by RNA Pol II, and the primary miRNAs (pri-miRNAs) are capped, spliced, and polyadenylated. RNase III-type enzyme Drosha cleaves long pri-miRNAs into precursor miRNAs (pre-miRNAs), and the resulting pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin. Another RNase III-type enzyme Dicer then processes the pre-miRNA to produce mature miRNA. The RNA-induced silencing complex (RISC), which includes TRBP and AGO I, complements target mRNAs for post-transcriptional gene silencing. (3)

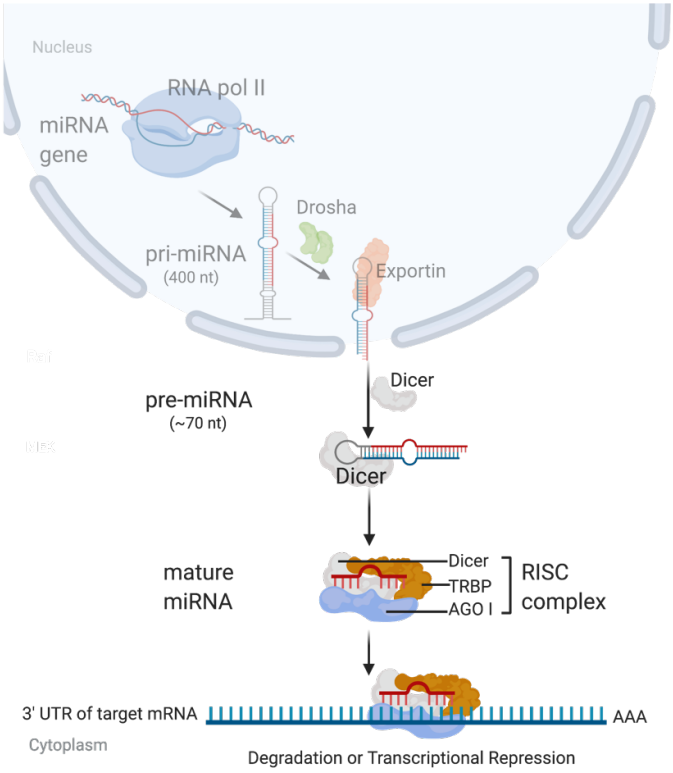


Figure 1.1 microRNA Biogenesis

The expression of miRNA is tightly regulated in normal cells, but dysregulation of miRNA expression has been observed in many types of cancer (oncogenic miRNA / oncomirs). The dysregulation of miRNA expression can cause some types of cancer including breast cancer, pancreatic cancer, prostate cancer, and lung cancer. (3-8) Several strategies have been developed to treat these cancers, by targeting the oncogenic miRNAs.

Krutzfeldt et al. developed antisense oligonucleotides (antagomirs), which are miRNA inhibitors that work by annealing to the mature miRNA's guide strand and inducing degradation or stoichiometric duplex formation, to block the overexpressed oncomirs. Their research focuses on improving the stability and specificity of antagomirs by adopting 2'-OMe-modified nucleotides with a phosphorothioate linkage that complements miR-122. (9,10) The antagomirs against miR-122 (antagomir-122) were administered to mice, and could decreased endogenous miR-122 level to undetectable level as long as 23 days post-injection. (11,12) Another strategy for inhibiting oncomir overexpression is genome editing using CRISPR/Cas9. Zhao et al used this strategy to downregulate miR-17-92 cluster and miR-21 by in vitro experiment. (13) Other researchers applied Cas9 mRNA and guide RNA into zebrafish embryos, as the result they reported chromosomal deletions and inversions. (14)

These strategies reported previously are shown to have adverse effects. Galbraith W.M., et al. reported that after administering antagomirs to monkeys, peripheral white blood cell counts decreased. Another issue raised by the use of antisense oligonucleotides is that these miRNA inhibitors may be unable to distinguish between members of the same family of miRNA. In contrast to the antisense oligonucleotide strategy, CRISPR/Cas9 gene-editing results in permanent gene modifications. However, the off-target effects have not been precisely profiled, this genome editing method must be improved further before being used as a substitute for cancer/disease therapy. (15,16,17)

Due to the challenges that come with those miRNA inhibitors, it may be useful to develop another strategy for inhibiting miRNA functions. Numerous strategies could be used to discover a novel method of inhibiting oncomirs overexpression. It is not necessary to develop inhibitors from scratch, rather, it is possible to repurpose currently available small molecules/drugs for miRNA inhibition by screening for the most suitable inhibitor candidates while also obtaining a drug candidate.

Target-based screening or biochemical assay is one of the strategies that are usually used if the target was identified. Coussens N.P. et al. describe this type of assay as having a variety of schemes, for example, to detect the binding between a small molecule and the target can be observed directly using fluorogenic products, or it can be coupled to a reporter that facilitates detection, such as luciferases. This biochemical assay is frequently used in combination with another analytical instrument, such as mass spectrometry, to quantify reaction products. (18-20)

Another screening approach is phenotypic assay. The advantage of target-based screening or biochemical assays is that they are more direct methods for quantifying or analyzing the effect of a small molecules on a target of interest, however, these strategies do not always reflect the biochemical processes in complex cellular environments. It is common for drug candidates to appear potent when tested in biochemical assays but fail to work in the cellular environment. Phenotypic assays cover a wide range of assessment techniques, from the simplest such as cell death or growth arrest to the use of animal models. The limitation of this type of assay is that it is mechanism-agnostic, which means that it cannot be used to determine the mechanism of action. (21-24)

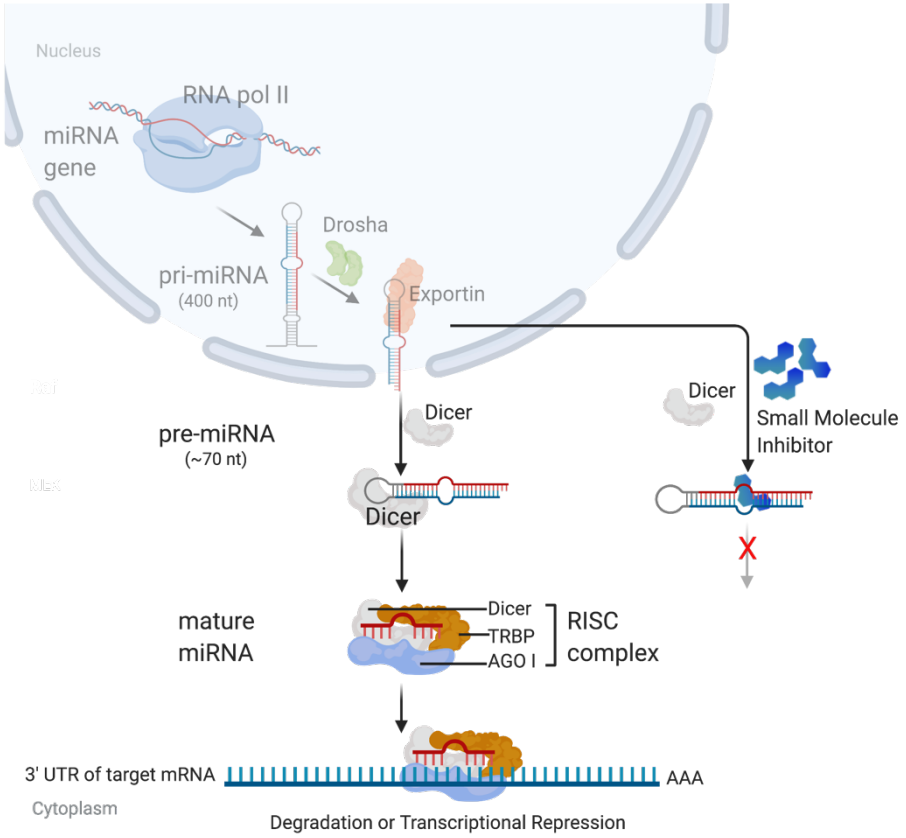


Figure 1.2 Dicer-mediated pre-miRNA maturation inhibition pathway

With the desire to find a new inhibitor of disease-associated miRNAs, the middle point of those two screening strategies would be preferred. We have attempted to target the biogenesis process of miRNAs by a small molecule (Figure 1.2), to inhibit the disease-associated miRNA. From the miRNA biogenesis process, we chose Dicer-mediated cleavage of pre-miRNA for a target-based screening. We also adopted a cell-based assay to see the effect of small-molecule on endogenous miRNA production. The adoption of a mechanism-specific assay, such as an in-vitro Dicer cleavage reaction, would resolve the phenotypic assay's mechanism-agnostic problem. On the other hand, adopting a cell-based assay would more reflect the biochemical processes in complex cellular environments. (25)

Applying those concepts to tackling the disease, we choose Uterine Corpus Endometrial Carcinoma (UCEC) as a cancer case model. UCEC is a malignant tumor of the female reproductive system that develops from the uterus's inner lining cells and is a major threat to women's health worldwide. (26,27) Additionally, UCEC accounts for approximately 20%-30% of female reproductive system cancers, ranking second only to cervical cancer. (28) Only a few drugs have been approved by the FDA for the treatment of this type of cancer. These drugs includes Pembrolizumab (Keytruda), Dostarlimab-gxly (Jemperly), Lenvatinib (Lenvima), and Megastrol (Megace). Due to the limited alternatives of available drugs, the discovery of novel drug candidates holds great promise for risk management and personalized therapeutic approaches for UCEC. (29)

A study by Favier A. et al. demonstrated a significant increase in the level of several miRNA expressions associated with this type of carcinoma, including miR-182, miR-31, and miR-30d. Similarly, the Cancer Genome Atlas (TCGA) revealed similar findings. In this study, we selected those three miRNAs as a model for implementing the combination of target-based and cell-based assay as described previously. (30)

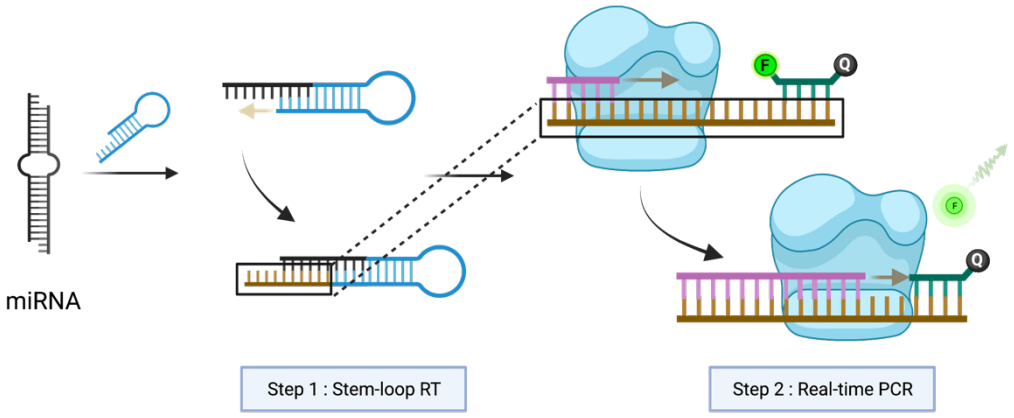


Figure 1.3 Stem-loop Reverse Transcription Real-Time PCR (RT-qPCR) Scheme

To evaluate miRNA production during the Dicer-mediated cleavage reaction, as well as miRNA expression during the cell-based assay, we used real-time PCR as the main instrument due to its fast and high-throughput detection and quantification of target nucleic acids sequences. (31) Due to specificity and sensitivity to detect and amplify the mature miRNA during reverse transcription (RT) reaction, stem-loop RT primer was used as illustrates in Figure 1.3. (32)

1.2 Sequence-Specific Small Molecules

Nakatani group has developed a number of sequence-specific DNA mismatch binding ligands (MBLs) that are capable of recognizing and binding to bulged sites and mismatched base pairs in duplex DNA (Figure 1.4). The developed molecules work by occupying the spaces and forming hydrogen bonds to the mismatched nucleotide bases. These ligands are then stabilized through stacking interactions with adjacent base pairs. (33)

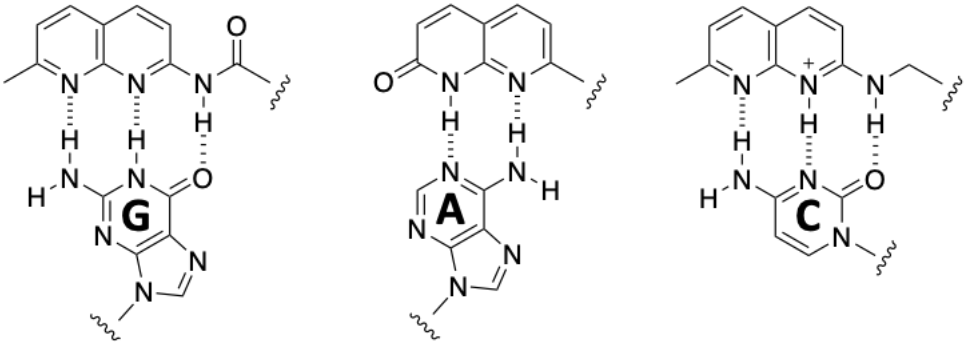


Figure 1.4 Nucleobase recognition parts. (a) Guanine recognition by *N*-Acyl-2-amino-7-methyl-1,8-naphthyridine, (b) Adenine recognition by 7-methyl-2-oxo-1,8-naphthyridine (azaquinolone), (c) Cytosine recognition by protonated 2-amino-7-methyl-1,8-naphthyridine

Naphtiridine Dimer (ND) is a dimeric form of *N*-acyl-2-amino-1,8-naphtiridine, which was the first compound designed to recognize G-G mismatches. From a structural perspective, this molecule was consists of two naphtyridine heterocycles connected by a linker. Two naphtyridine form hydrogen bonding with the guanine, and the linker that contained a secondary amino group offers the appropriate conformational restriction to the dynamic motion of two heterocycles besides providing attractive electrostatic interaction to negatively charged DNA and gained water solubility. (34-36)

8-azaquinolone, which is a complement to adenine in terms of its hydrogen bonding surface was utilized to develop another molecule that could recognize G-A mismatches, naphtiridine-azaquinolone (NA). In another case, a hydrogen bonding rearrangement of the donor-donor-acceptor groups of cytosine was required to develop C-C mismatch recognition, which was achieved through the development of a protonated form of 2-amino-1,8-naphtiridine. This molecule meets the requirement that needed, and it is also reported that 2-amino-1,8-naphtiridine is strongly and selectively bound to C-C mismatch. (37-40)

Due to the specific nucleobase recognition properties of the small molecules synthesized and developed by Nakatani group, it is highly likely that those small molecules will bind to pre-miRNAs that has the unpaired nucleotides in their structure, which may affect the miRNA maturation process. If small molecules could inhibit UCEC-associated oncogenic miRNA production *in vitro* and also in cells, then those small molecules have the potential to be drug candidates.

I.3 Objectives

The objective of this research is to analyze the effect of the small molecule on the Dicer-mediated cleavage of pre-miRNA *in vitro* and in cells, seeking for potential small-molecule candidates for treating UCEC that are associated with miRNA dysregulation. To achieve that objective, we demonstrated a combination of assays between target-based assay and cell-based assay to screen the small molecules that shows the potential inhibitory activity to mature miRNA production.

Aside from that, the results and the screening methods from this research could be used for consideration in the development of a high throughput screening system of the small molecules that targeting UCEC-associated miRNA.

CHAPTER II
Utilization of Real-Time PCR to determine Kinetical Properties of In-vitro Dicer reaction

2.1 Introduction

A small molecule that binds to a specific pre-miRNA and inhibits or accelerates its processing can be a tool for modulating miRNA-mediated gene regulation. It was previously reported that by protonating the nitrogen in the 2,7-diamino-1,8-naphthyridine chromophore, N,N'-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP) could form hydrogen bonds with cytosine as shown in Figure 2.1 A. (39) This compound, however, has a limitation in that it does not bind to RNA. To address this issue, this compound was modified by the addition of an expanded ring system (BzDANP) (Figure 2.1 B), which may improve stacking interaction with neighboring base pairs in RNA. (40)

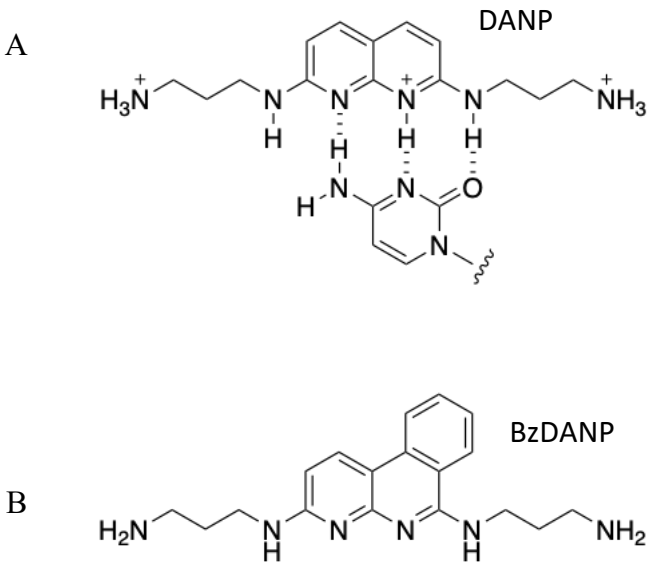


Figure 2.1 DANP could form hydrogen bonding with cytosine (A) and BzDANP (B)

Further studies of BzDANP by Otabe, et.al shows that BzDANP reduced the initial velocities of the pre-miR-136 Dicer cleavage reaction, resulting in a significant decrease in Vmax.(41) BzDANP has the potential to be used in other pre-miRNAs, but one of the major

challenges is determining the kinetical properties of this compound in Dicer-mediated cleavage reactions in a more direct and convenient way than isotope labeling. One option is to directly detect the product, which is miRNA, which can be done with real-time PCR. Here we conducted an experiment to determine the kinetical properties by using real-time PCR.

2.2 Results and Discussions

To determine the kinetical properties of *in vitro* dicer reaction, several different concentrations of substrates (pre-miR-136) were used, ranging from 25 nM to 400 nM, as well as different incubation times at the in vitro dicer reaction. The aliquots of the reaction solution separated after 2.5, 5, 10, 20, 40, 80, and 180 minutes incubation. Figure 2.2 and 2.3 shows the correlation between concentration miR-136 product and time for every variation concentration of substrate.

Table 2.1 shows every initial velocity from various substrate concentrations. The initial velocity then was plotted against substrate concentrations and the obtained plots were analyzed by least-squares fitting to the Michaelis-Menten equation. The Michaelis-Menten analysis for the *in vitro* dicer reaction of pre-miR-136 yielded a *Km* of 9.71 x 10^{15} nM, and V_{max} 4.31 x 10^{19}nM min^{-1}. The results obtained here are far different compared to the previous study by Otabe, et.al, in which they obtained Km of 52.6 $\pm$ 8.6 nM and Vmax of 1.52 $\pm$ 0.08 nM min^{-1}.

The differences between these two result raised from the different methods that used. It is important to note that using qPCR as an instrument to measure the kinetical aspect especially in this *in-vitro* dicer reaction is still needs to be improved. Several critical points often occur during the experiments, the technical handling problem quite often becomes a problem due to multi-step procedures, from the sampling, preparation for reverse transcription, and the preparation for real-time PCR, especially for the standard curve technique. This makes the results are often difficult to reproduce in the same quality. Unfortunately, because of the limitation of time to overcome this problem, it is difficult to continue this experiment.

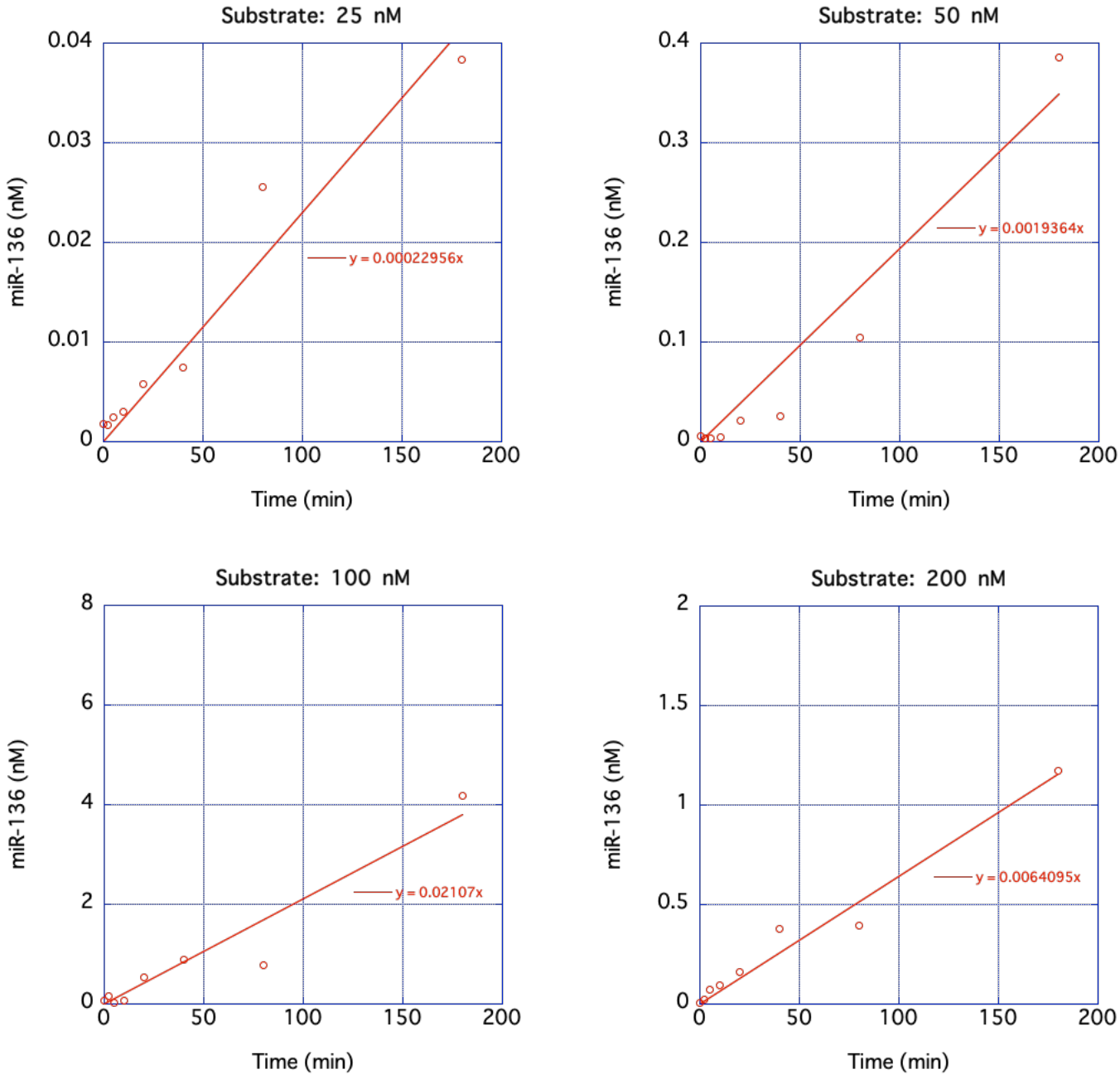


Figure 2.2 Velocity plot of *in vitro* Dicer reaction by several different substrate concentration

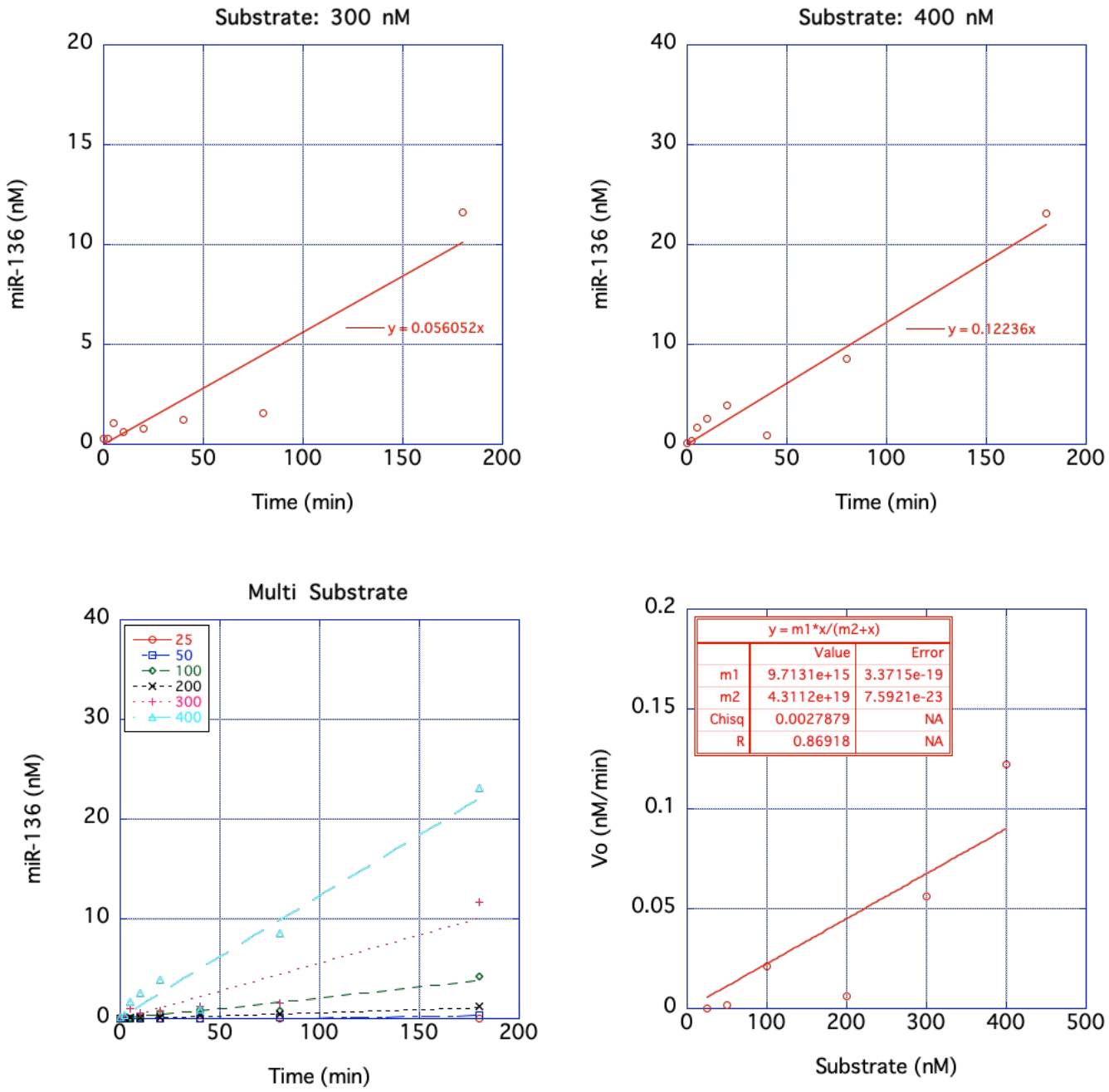


Figure 2.3 Velocity plot of *in vitro* Dicer reaction by several different substrate concentrations

Table 2.1 Initial Velocity of several different substrate concentrations

pre-miR-136 (nM)		25	50	100	200	300	400
Vo	**(nM/min)**	0.0002	0.0019	0.0211	0.0064	0.0561	0.1224

2.3 Conclusion

We have demonstrated the utilization of real-time PCR to investigate the kinetical properties of the *in vitro* dicer reaction. Compared to the previous study conducted by Otabe, et.al, we obtain significantly different results. As mentioned in the results and discussion section, the methods for utilizing real-time PCR to investigate the kinetic properties of enzymatic reactions particularly those used in the *in vitro* dicer reaction still require improvement. It would be preferable to reduce the systematical error aas possible, for example by using one-step RT-qPCR reagents, or digital droplet PCR which eliminates the need to create a standard curve.

2.4 Methods

2.4.1 Time-dependent Dicer Cleavage Reaction

In the presence of a buffer reaction, phosphorylated precursor microRNA (25 nM), 50 mM $MgCl_2$, 10 mM ATP, 0.05 percent BSA, and Dicer (50 nM) were mixed. 6 different variation concentration (25, 50, 100, 200, 300, 400 nM) of BzDANP was then added, with RNAse-free water serving as a negative control. After that, the mixture was incubated at 37^{o}C for several variation time (0, 2.5, 5, 10, 20, 40, 80, 180 minutes). The reaction was then halted by adding 10 mM EDTA.

2.4.2 Reverse Transcription Real-time PCR (RT-qPCR)

The 100 mM dNTP Mix, the Multiscribe Reverse Transcriptase, and the 5X Reverse Transcription primer were combined and incubated according to the manufacturer's protocol. After that, the mixture was mixed with the Taqman Master Mix. This mixture was then loaded to the real-time PCR instrument, and the resulting data were analyzed using the real-time PCR instrument's software.

CHAPTER III
Study of Mechanism of Action (MOA)-based Screening Assay of Small Molecules Targeting Uterine Corpus Endometrial Carcinoma (UCEC)-associated microRNA

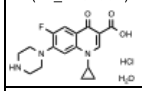
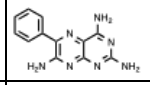
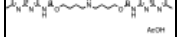
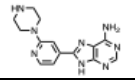
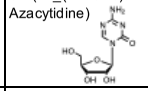
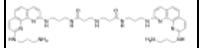
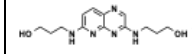
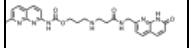
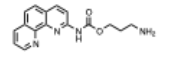
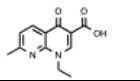
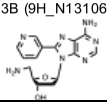
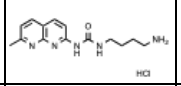
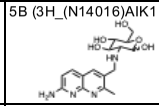
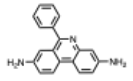
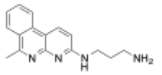
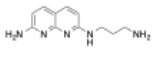
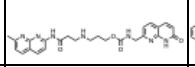
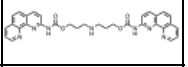
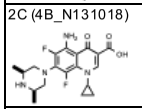
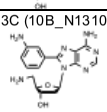
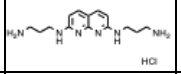
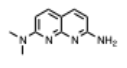
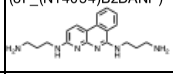
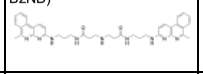
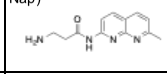
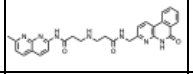
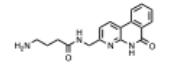
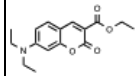
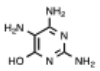
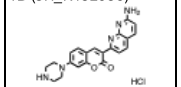
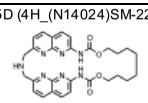
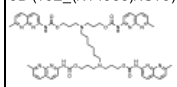
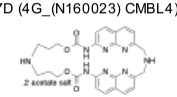
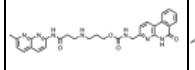
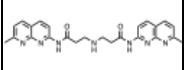
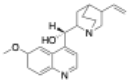
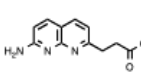
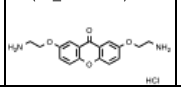
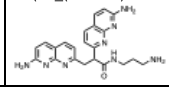
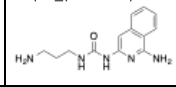
3.1 Introduction

UCEC is a malignant tumor of the female reproductive system that develops from the cells of the uterus's inner lining. It is a major threat to women's health globally. (26,27) Additionally, UCEC accounts for approximately 20%-30% of female reproductive system cancers, trailing only cervical cancer in this category. (28)

Because the available drugs to treat this type of cancer are still limited, it would be preferable to discover a new drug to treat this type of cancer. From a mechanism of action standpoint, the majority of drugs used to treat UCEC were either PD-1 binders or kinase inhibitors. For this reason, an alternative to searching for a new drug is to identify a potential pathway associated with this cancer that could be used as a starting point for drug discovery.

In this chapter, the screening of small molecules to find the UCEC drug candidates was demonstrated. All of the small molecules that used in this screening shows in Figure 3.1. MicroRNA biogenesis was adopted into the screening system to mimic the Dicer-mediated pre-miRNAs maturation process. This process is conducted in-vitro as previously mentioned as target-based screening. (18,19) The compounds obtained from this screening then continued to the in-cell screening, to know the effect of the small molecules in the living cells environment.

Further analysis was then performed on the small molecules that may possess the potential oncogenic miRNA inhibitors, such as SPR analysis to analyze the small molecule's binding affinity to the pre-miRNA and its viability, which measures the effect such as IC_{50}.

2A (2B_N131002) 	3A (8D_N131052) 	4A (5B_N132026) 	5A (2B_(N14002)AIK041) 	6A (7F_(N14046)5-Azacytidine) 	7A (3C_(N160011) DDAP) 	8A (7H_(N160048) AIK248) 	9A (7A_(N14241)ljx106) 	10A (8H_(N14256)ljx305) 
2B (2H_N131008) 	3B (9H_N131064) 	4B (5C_N132027) 	5B (3H_(N14016)AIK112) 	6B (8A_(N14049)otb003) 	7B (4B_(N160018) Am-BzN) 	8B (8D_(N160052) ih 039) 	9B (7B_(N14242)ljx249) 	10B (9A_(N14257)ljx280) 
2C (4B_N131018) 	3C (10B_N131066) 	4C (6F_N132038) 	5C (4B_(N14018)SA029) 	6C (8F_(N14054)BzDANP) 	7C (4C_(N160019) Am-BzND) 	8C (9A_(N160057) bAla-Nap) 	9C (7C_(N14243)BzNA) 	10C (9B_(N14258)ljx188) 
2D (4G_N131023) 	3D (11H_N131080) 	4D (8H_N132056) 	5D (4H_(N14024)SM-221) 	6D (10E_(N14069)NCT6) 	7D (4G_(N160023) CMBL4) 	8D	9D (7D_(N14244)ljx253) 	10D (9F_(N14262)ljx289) 
2E (6A_N131033) 	3E (2A_N132001) 	4E (9F_N132062) 	5E (5C_(N14027)YM011) 	6E (2B_(N160002) 5H-iQ) 	7E (5A_(N160025) CMBL5a)	8E	9E (7E_(N14245)ljx107)	10E (9H_(N14264)ljx372)
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2G (6G_N131039)	3G (4E_N132021)	4G (11F_N132078)	5G (5G_(N14031)NN246)	6G (2G_(N160007) PQA-C3-amide)	7G (6C_(N160035) NCD-C3-NH2)	8G	9G (8A_(N14249)ljx238)	10G (10B_(N14266)ljx344)
2H (7C_N131043)	3H (4H_N132024)	4H (11H_N132080)	5H (6G_(N14039)Thioflavin)	6H (3B_(N160010) PQAD-urea)	7H (7F_(N160046) AIK216)	8H	9H (8B_(N14250)ljx223)	10H (10D_(N14268)ljx358)

Figure 3.1 67 Library compounds from Nakatani Laboratory group

3.2 Results and Discussions

3.2.1 *In Vitro* Dicer Reaction Screening

To determine the effects of small molecules in the dicer processing reaction, we performed *in vitro* dicer reaction by using pre-miR-182, pre-miR-31, and pre-miR-30d as the miRNA targets and 67 selected small molecules from the Nakatani laboratory library as tested compounds. The products of this reaction were either promoted or inhibited mature miRNA. However, to measure the product we need to perform a reverse transcription reaction to transcribe miRNA products to cDNA. This cDNA was then quantified using qPCR.

Figure 3.2 , 3.3, and 3.4 shows the qPCR results of three different miRNAs, each of which was replicated twice. The $2^{-\Delta CT}$ expressions were used to evaluate the amount of cDNA that is directly proportional to the amount of miRNA products affected by each tested compound. For every compound that shows the $2^{-\Delta CT}$ value greater than 1, this indicates that the compound promoted miRNA production, on the other hand, any compound with $2^{-\Delta CT}$ value less than 1 inhibits miRNA production during the dicer processing reaction. To screen the compounds that would be used in the next step of screening, we introduced a $2^{-\Delta CT}$ value of 0.5 as a threshold which is analogous to IC_{50} which indicates that certain compounds inhibited 50% of miRNA production. In this case, a lower $2^{-\Delta CT}$ value indicates greater inhibition which is preferable. Aside from $2^{-\Delta CT}$ expressions, the replication was also considered when selecting compounds for the next step of the screening, only compounds that passed the threshold twice were chosen for the next screening.

According to the criteria described above, Figure 3.2 demonstrates that 26 compounds pass the 0.5 threshold and also meet the replication criteria, indicating that those 26 compounds may exhibit inhibitory activity against the dicer-mediated pre-miR-182 processing reaction. The inhibition of pre-miR-31 and pre-miR-30d processing reaction shows in Figure 3.3 and 3.4, there are 31 and 22 different compounds that have shown potential inhibitory activity to the pre-miR-31 and pre-miR-30d.

The in vitro dicer reaction result was summarized in Figure 3.5, which shows 48 different small molecules inhibiting three different dicer-mediated pre-miRNA processing. All of it could be divided into three categories: compounds that appear to preferentially inhibit

one type of miRNA, compounds that appear to preferentially inhibit two types of miRNAs, and compounds that appear to preferentially inhibit all three types of miRNAs.

Following that, we performed in-cell screening to investigate the biological significance of those 48 compounds in living cells.

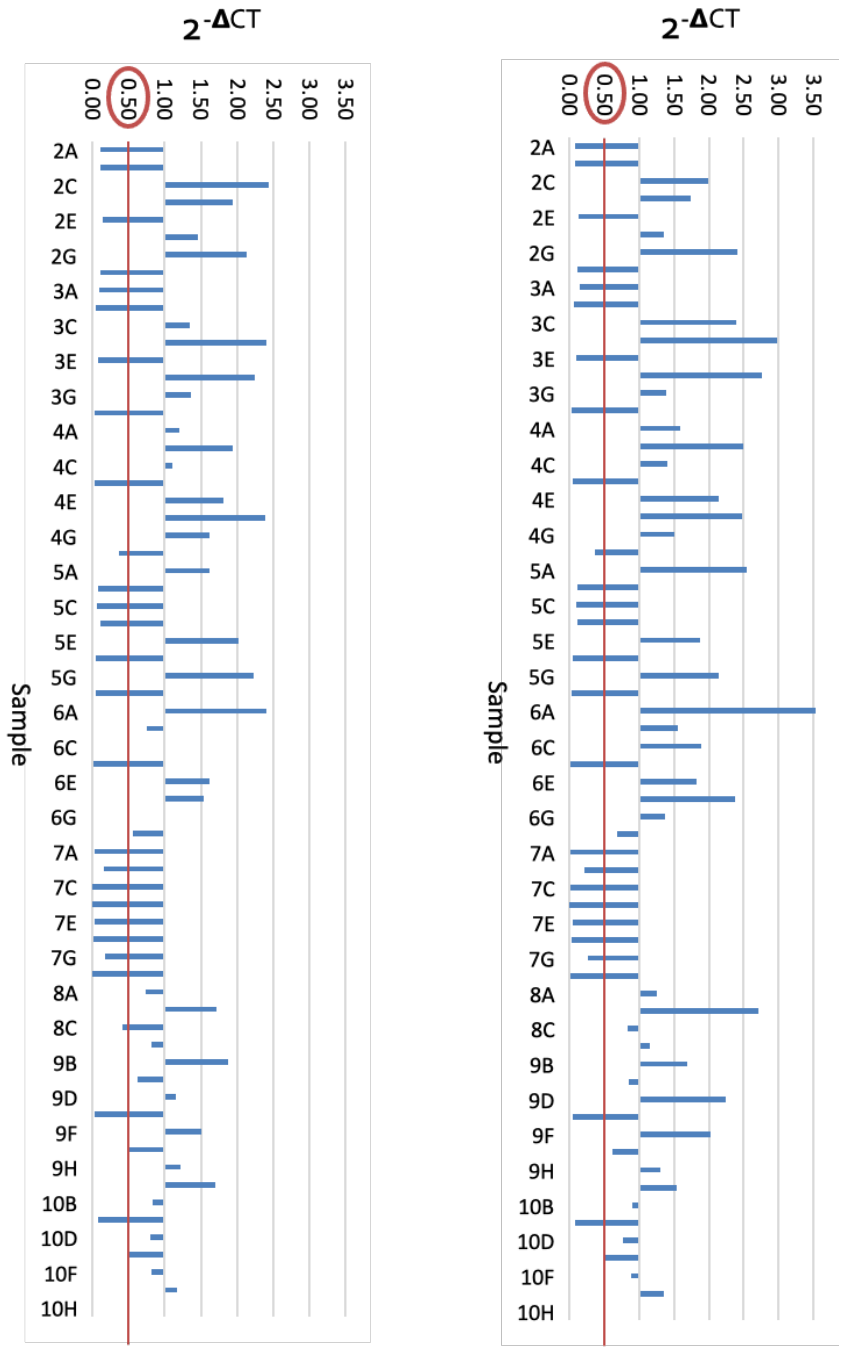
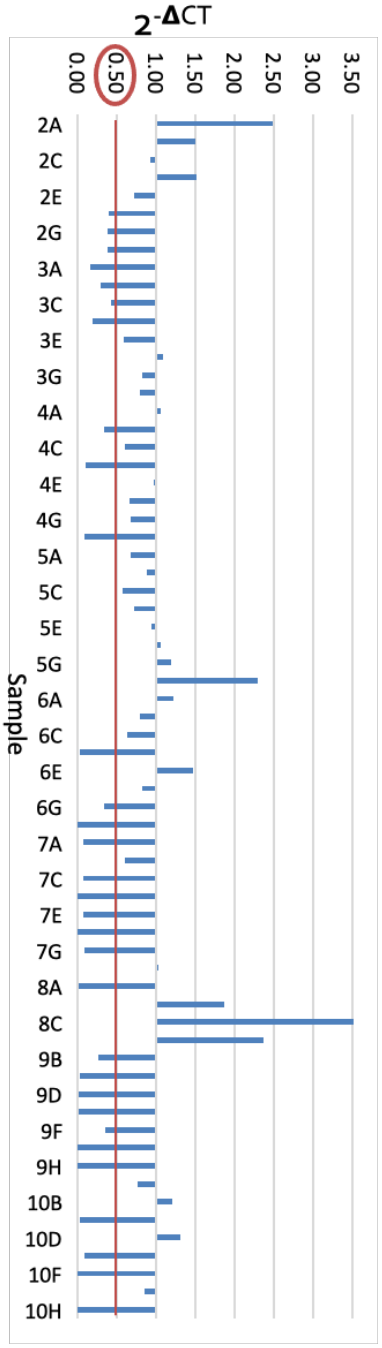


Figure 3.2 *In vitro* Dicer screening of hsa-miR-182



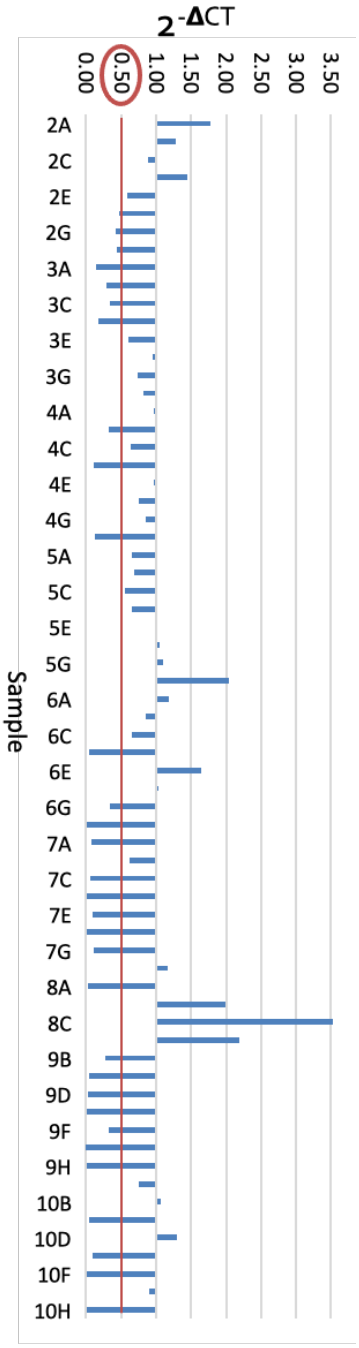


Figure 3.3 *In vitro* Dicer screening of hsa-miR-31

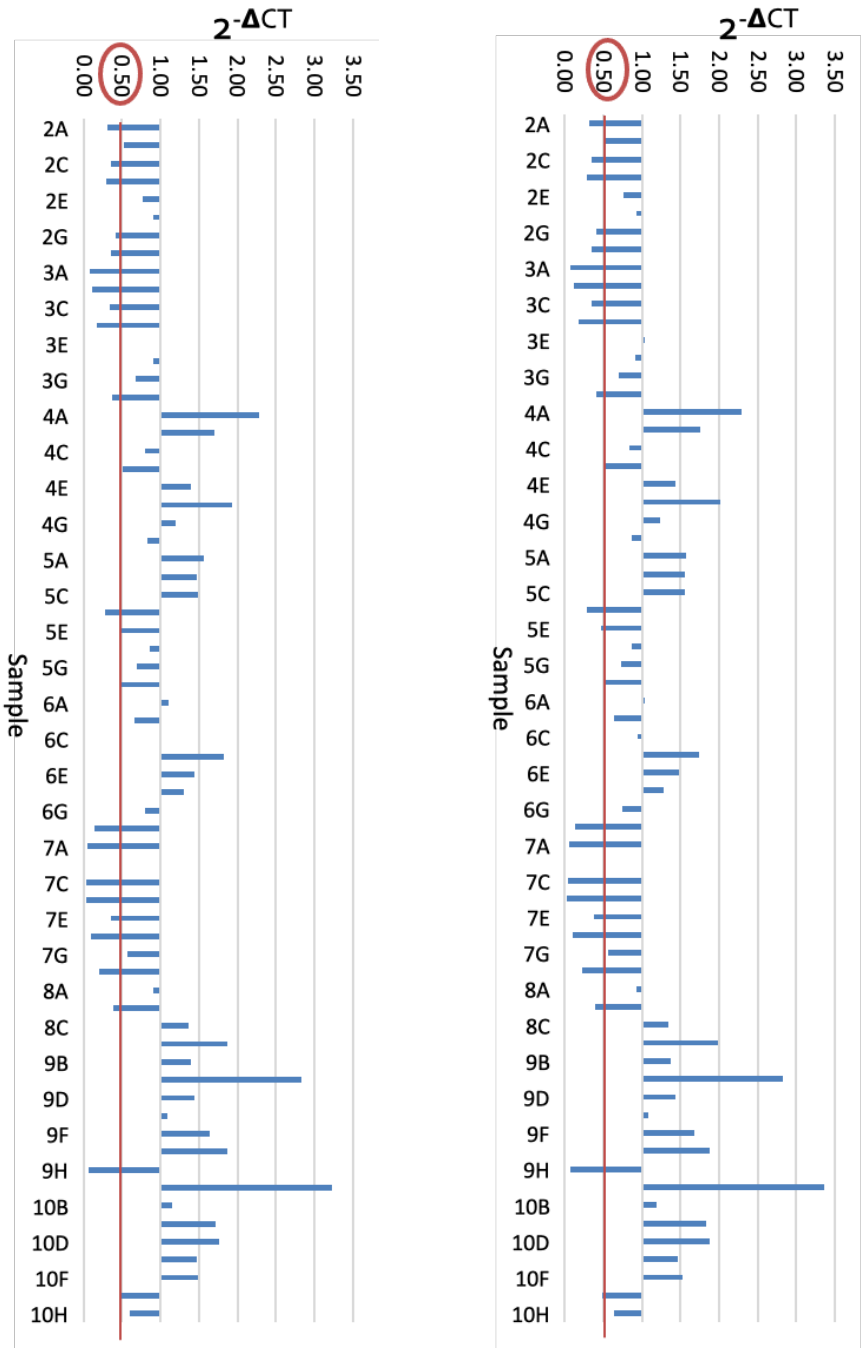
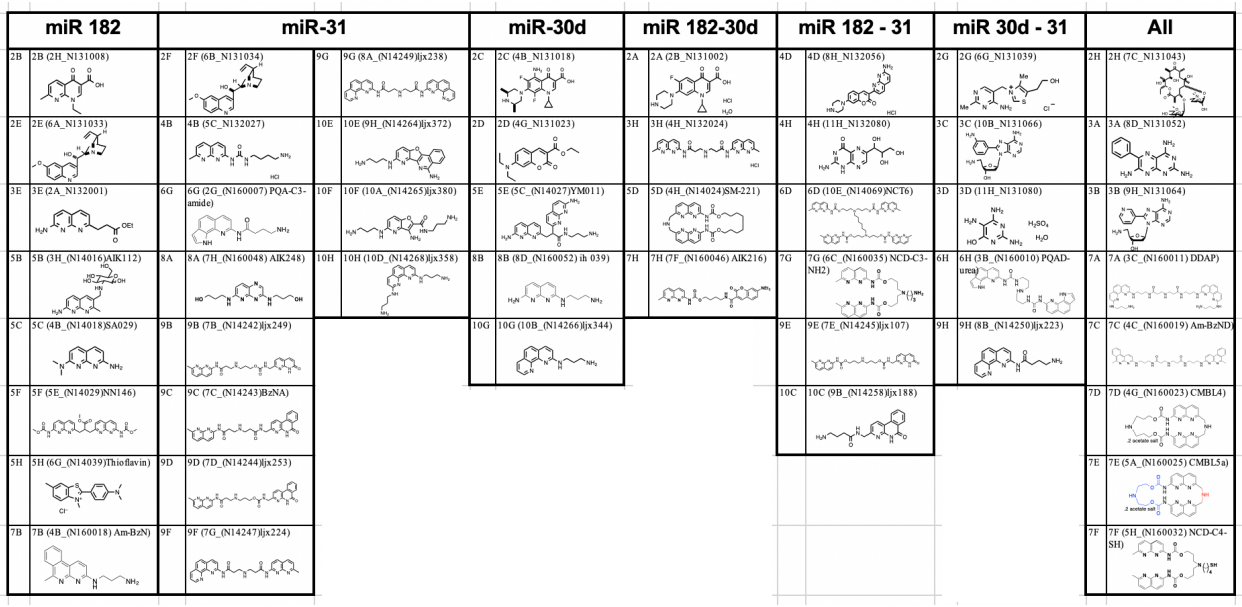


Figure 3.4 *In vitro* Dicer screening of hsa-miR-30d



microRNA	compound that inhibit
miR-182	**8**
miR-31	**12**
miR-30d	**5**
miR-182 and miR-30d	**4**
miR-182 and miR-31	**6**
miR-31 and miR 30d	**5**
miR-182-31-30d	**8**
Total	**48**

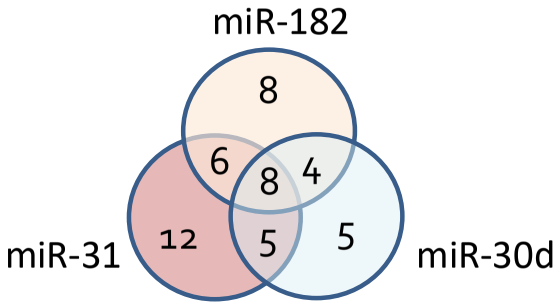


Figure 3.5 *In vitro* Dicer screening results and its summary

3.2.2 Feasibility Assay of HeLa cell-line

In order to confirm the feasibility of HeLa cells as a model organism in this study, we examined the expression of three types of miRNA (hsa-miR-182, hsa-miR-31, and hsa-miR30d) in these cells. The HeLa cells were cultured and lysed using standard technique, and the miRNAs of interest were quantified by applying RT-qPCR. We found that the average CT value of all three types of miRNAs was less than 30 (out of 40) as shown in Table 3.1, implying that the difference in value before and after the addition of the compounds could be easily counted, and we concluded that HeLa cells are viable to use in this study.

	Replication	microRNA		
		182	31	30d
CT Value	I	27.276	20.357	24.583
	II	25.926	20.295	24.928
	III	26.625	20.178	25.307
	Average	**26.609**	**20.277**	**24.939**

Table 3.1 CT value summary of miR-182, miR-31, and miR-30d in HeLa cells

3.2.3 In-Cell Screening

To investigate the effects of previously-screened small molecules on the expression of hsa-miR-182, hsa-miR-31, and hsa-miR-30d in living cells, we added those compounds to the cells and incubated them overnight to reveal their biological effects. After incubation, the cells were lysed, and the expression of miRNAs was quantified using RT-qPCR techniques similar to the *in vitro* dicer reaction.

Figure 3.6, 3.7, and 3.8 shows the expression of hsa-miR-182, hsa-miR-31, and hsa-miR-30d on the triplication of each miRNA, respectively. In contrast to the *in-vitro* dicer reaction, to evaluate the amount of cDNA products, we used $2^{-\Delta\Delta CT}$ expressions rather than $2^{-\Delta CT}$ because of the differences in normalization calculation between the two. The CT value of compound-affected miRNA was normalized to the CT value of the untreated product in terms of *in-vitro* dicer reaction. In the cell-based assay, we used two-step normalization, first normalizing the CT value of treated cells to the CT value of untreated cells, then normalizing it again to the internal control gene, in this case, U6 snRNA. Even though the expression used is different, the practical definition of the two is the same. If the $2^{-\Delta\Delta CT}$ expressions value is less than 1, there is an inhibition effect on miRNA expression and vice versa.

To determine which compounds exhibit potential inhibitory activity, we set the $2^{-\Delta\Delta CT}$ expressions value of 0.5 as a threshold, and compounds with $2^{-\Delta\Delta CT}$ values less than the threshold have significant inhibition effects. Similar to the screening criteria in the previous *in vitro* dicer reaction screening, we considered replication in addition to the $2^{-\Delta\Delta CT}$ expressions value. If a compound showed the $2^{-\Delta\Delta CT}$ expressions value less than the threshold more than or equal to two times replication, we considered this compound as a potential inhibitor.

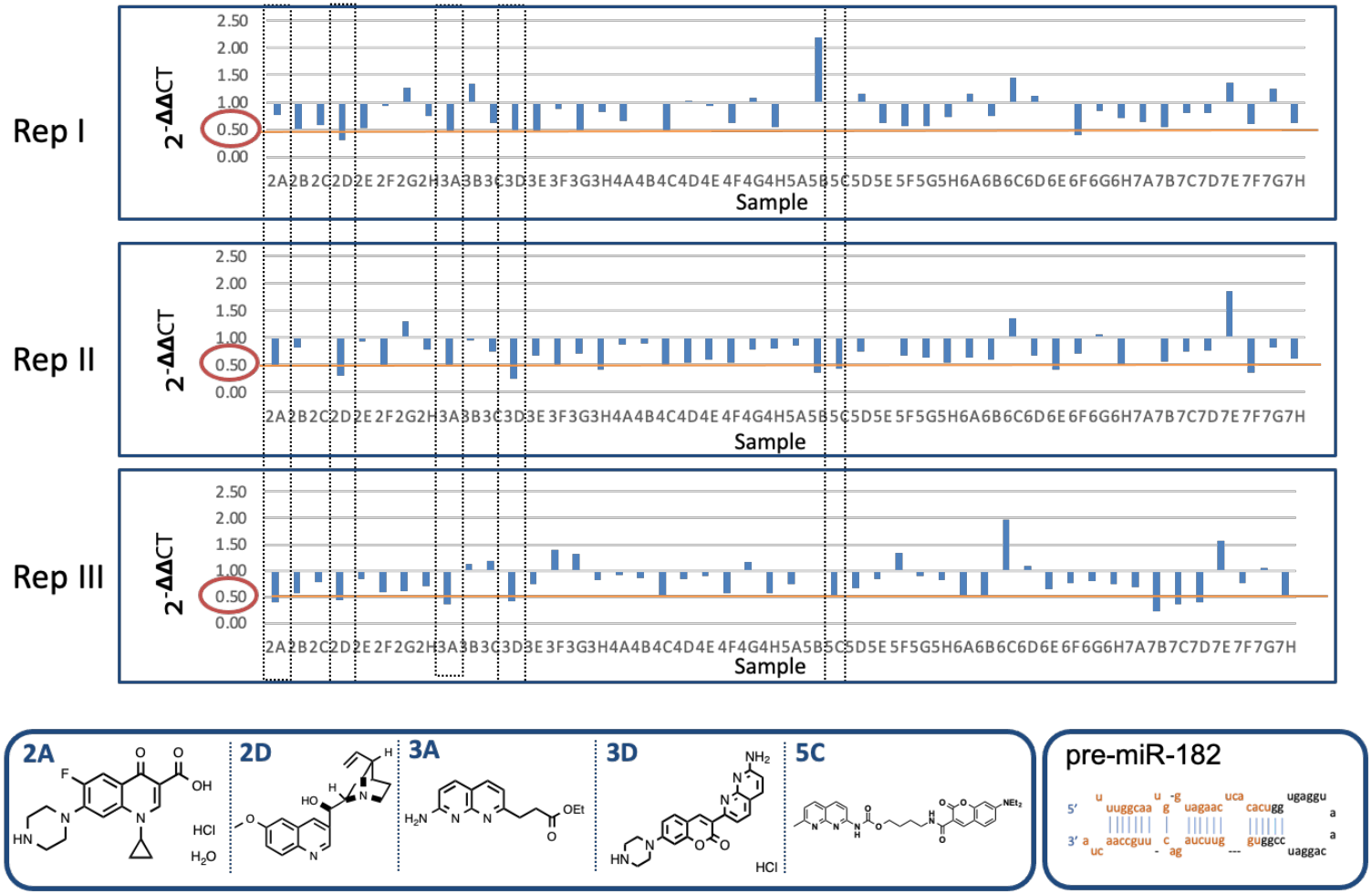


Figure 3.6 *In-Cell* screening results of hsa-miR-182

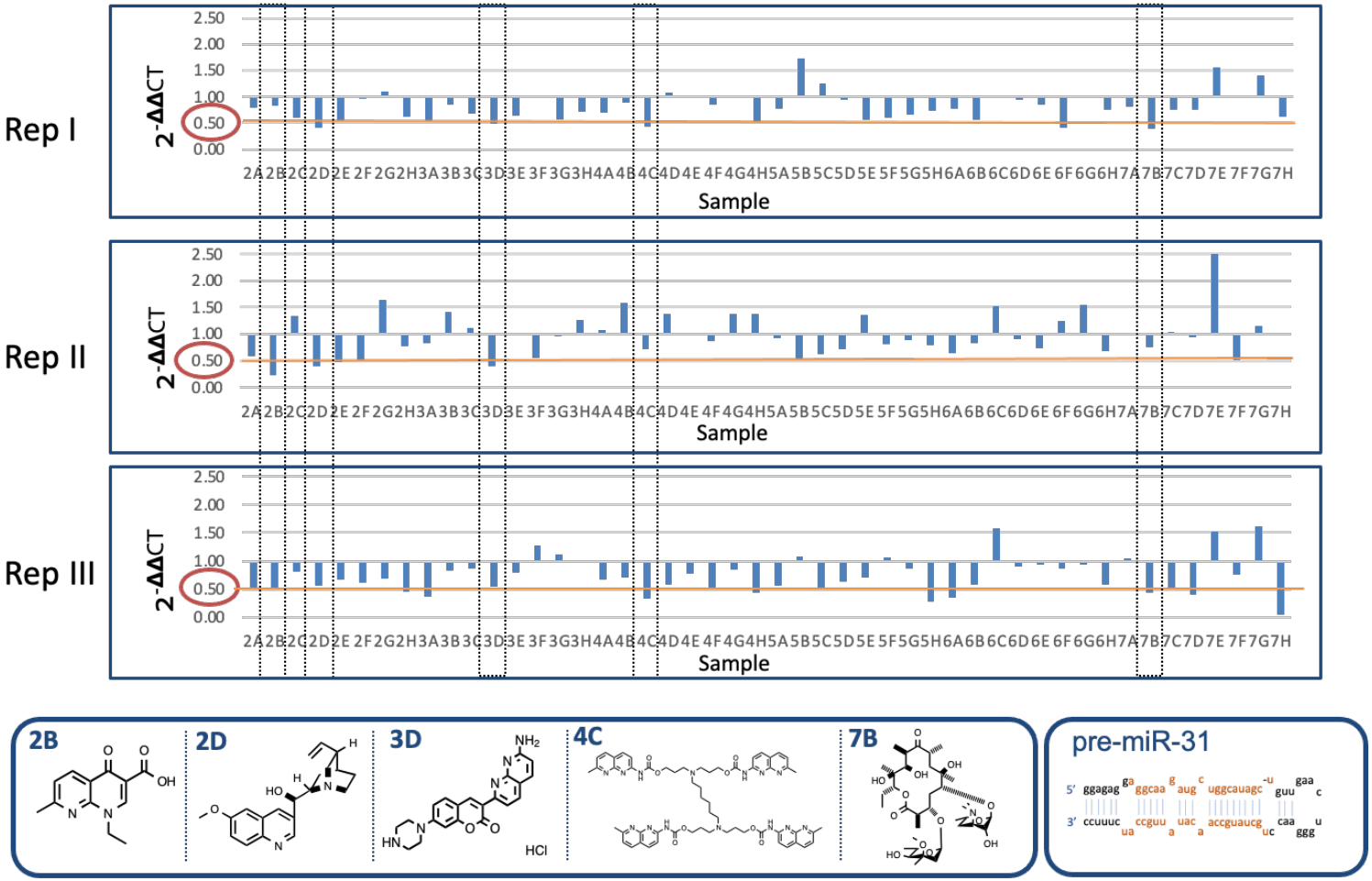


Figure 3.7 *In-Cell* screening results of hsa-miR-31

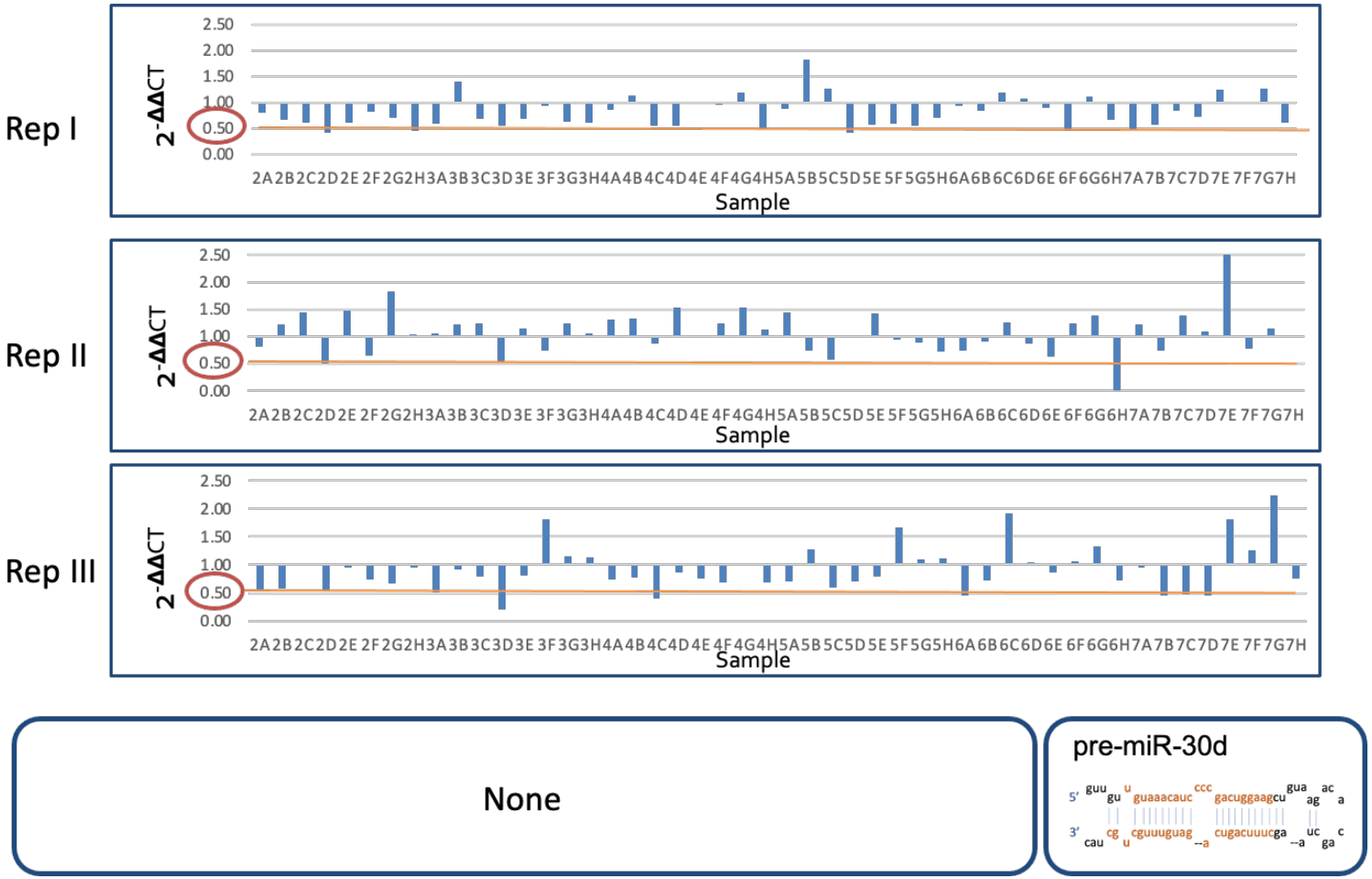


Figure 3.8 *In-Cell* screening results of hsa-miR-30d

According to the criteria provided above, Figure 3.6 demonstrates that 5 compounds reach the 0.5 threshold and also meet the replication criteria, indicating that these 5 compounds may possess inhibitory activity against the pre-miR-182 processing reaction. The inhibition of pre-miR-31 and pre-miR-30d processing reaction shows in Figure 3.7 and 3.8. While 5 different compounds have demonstrated potential inhibitory activity against hsa-miR-31 production, there are no compounds that meet all of the criteria listed above for has-miR-30d production.

The summary of compounds that inhibit hsa-miR-182 and hsa-miR-182 production is shown in Figure 3.9. Compounds 2A, 3A, and 5C inhibit the production of has-miR-182, whereas compounds 2B, 4C, and 7B inhibit the production of hsa-miR-31. In contrast to the other six compounds, which appear to preferentially inhibit one type of miRNA, compounds 2D and 3D inhibit both types of miRNA production.

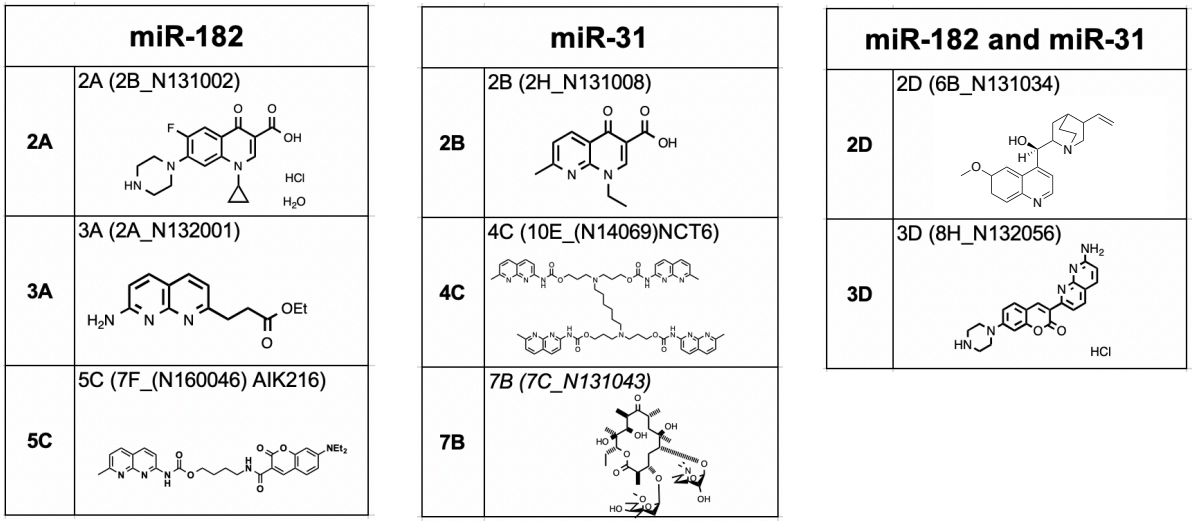


Figure 3.9 *In-Cell* screening results summary

3.2.4 Confirmation Experiment *In Cell* Reaction

To verify the validity of the criteria used in the *in vitro* dicer reaction screening, we performed a similar experiment on the 19 compounds that were excluded from the in-cell screening. These compounds were used to treat HeLa cells in a similar manner, and the results of this experiment were evaluated similarly to those from the in-cell screening.

As presented in Figure 3.10, 3.11, and 3.12, none of the compounds significantly inhibited has-miR-182, has-miR-31, or has-miR-30d production by failing to pass the $2^{-\Delta\Delta CT}$ expressions threshold, and even when several compounds did pass the threshold, those compounds did not meet the replication criteria, defined as passing the threshold more than twice. The validity of the screening criteria is concluded as a result of these findings.

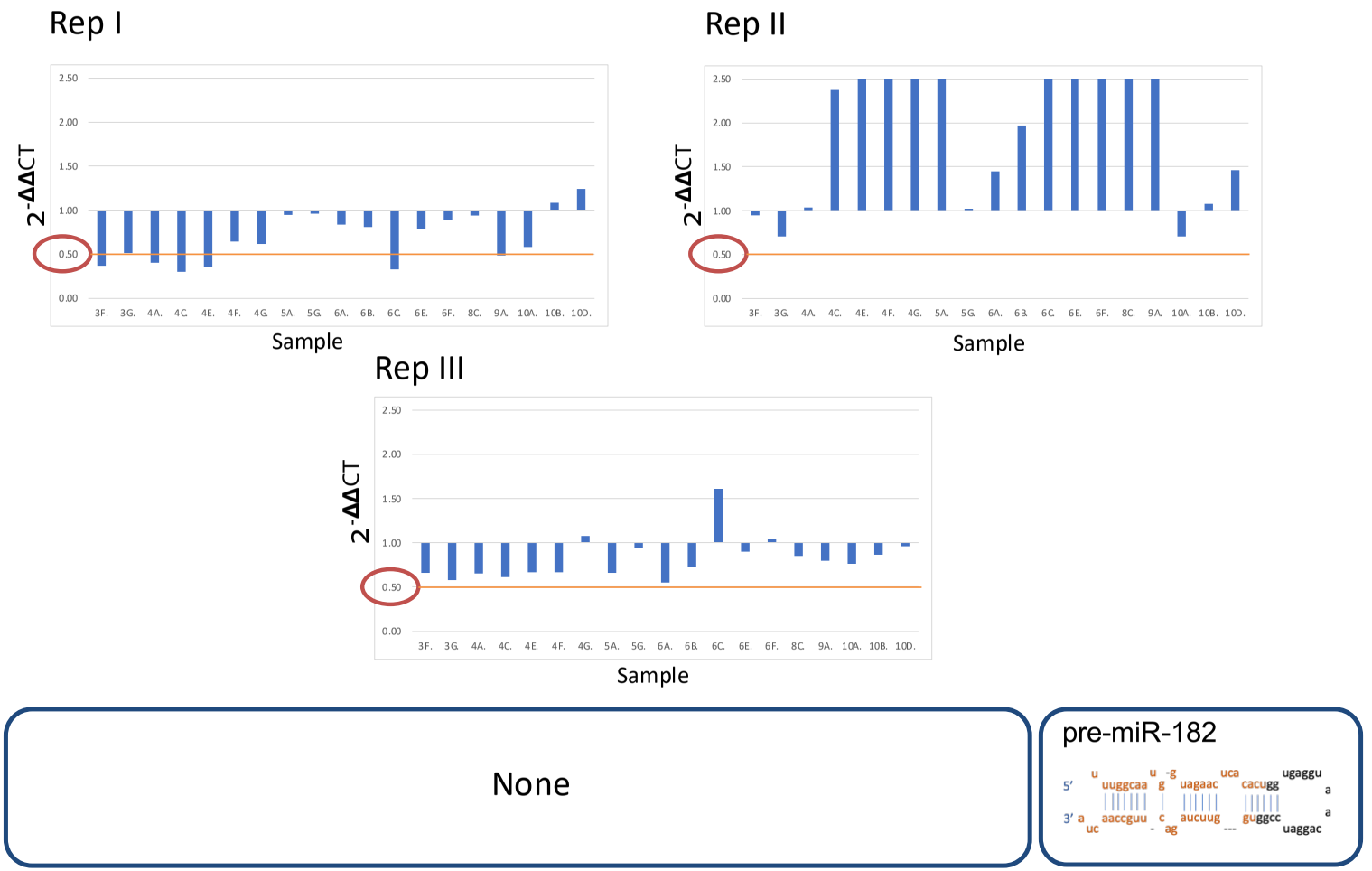


Figure 3.10 In cell assay of 19 compounds that excluded after first screening to confirm the expression of hsa-miR-182. This results indicates that none of the compounds that pass the threshold and meet the replication criteria

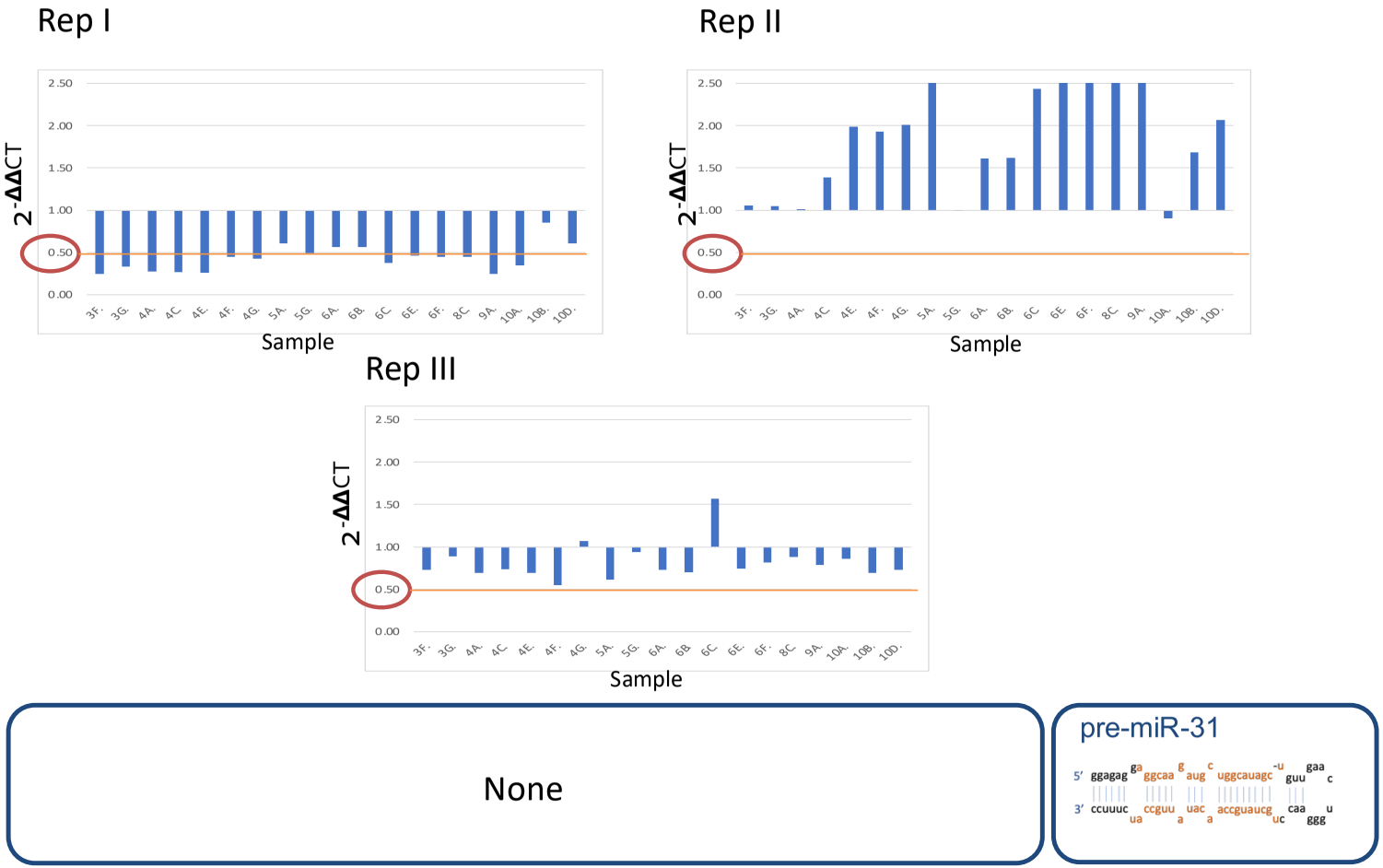


Figure 3.11 In cell assay of 19 compounds that excluded after first screening to confirm the expression of hsa-miR-31. This results indicates that none of the compounds that pass the threshold and meet the replication criteria

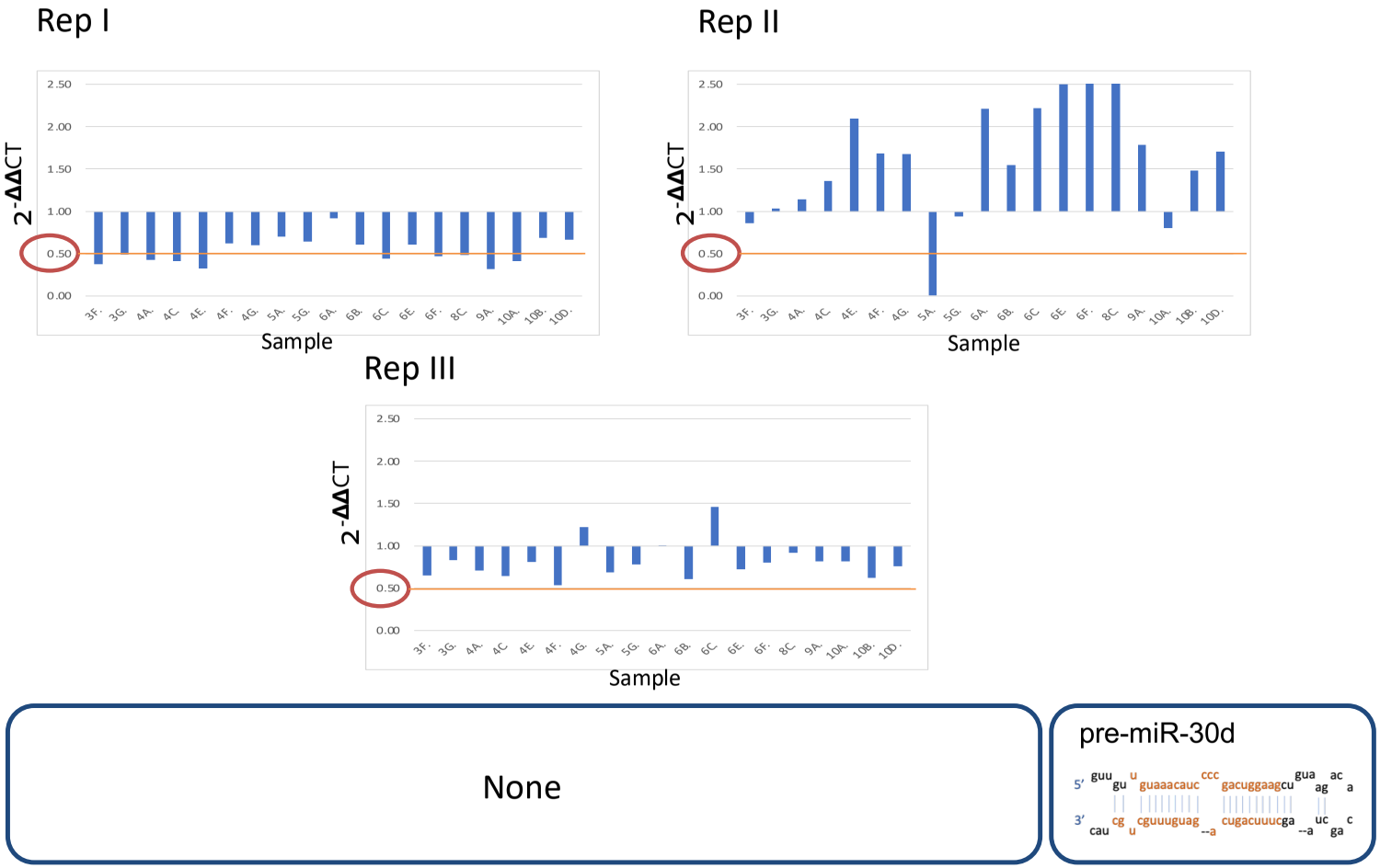


Figure 3.12 In cell assay of 19 compounds that excluded after first screening to confirm the expression of hsa-miR-30d. This results indicates that none of the compounds that pass the threshold and meet the replication criteria

3.2.5 Surface Plasmon Resonance (SPR) Analysis

It was previously reported that the small molecule could bind to precursor miRNAs and then interfere with the cleavage processing of the dicer. (41) To verify this possibility, SPR analysis was used to determine the mechanism of action (MOA) of the small molecules that inhibited hsa-miR-182, hsa-miR-31, and hsa-miR-30d production. This study will assess the binding ability of 8 small molecules identified through in-cell screening (2A, 2B, 2D, 3A, 3D, 4C, 5C, and 7B) to the precursors of hsa-miR-182, hsa-miR-31, and hsa-miR-30d.

Binding experiments were generally carried out on the SA chip with amounts of immobilized pre-miR-182, pre-miR-31, and pre-miR-30d corresponding to 1606.8, 2150.0, and 1950.7 RU, respectively. Figure 3.13 and 3.14 depicts typical binding curves monitored during contact time with an 8-compound solution. It demonstrates that compounds 3D and 4C have better binding profiles than the other six compounds. A comparison of compound 3D and 4C binding to pre-miRNAs reveals that pre-miR-182 has the highest response, followed by pre-miR-31 and pre-miR-30d, respectively.

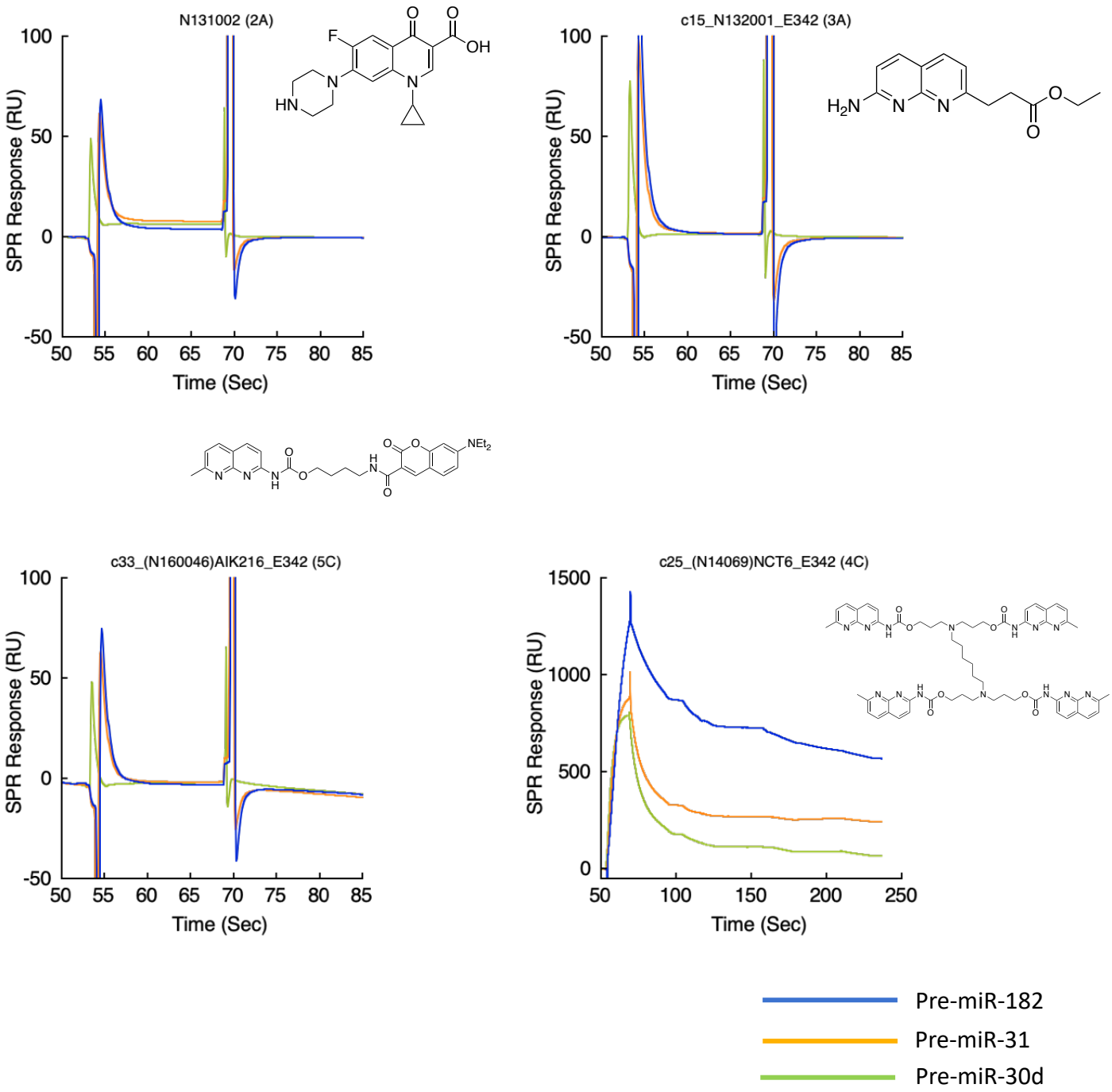


Figure 3.13 SPR binding assay of compound 2A, 3A, 5C, and 4C to the pre-miR-182/31/30d immobilized surface. Ligand was added at concentration of 50 uM. The amount of pre-miR-182/31/30d immobilized on SA chip was 1606.8, 2150, 1950.7 RU respectively

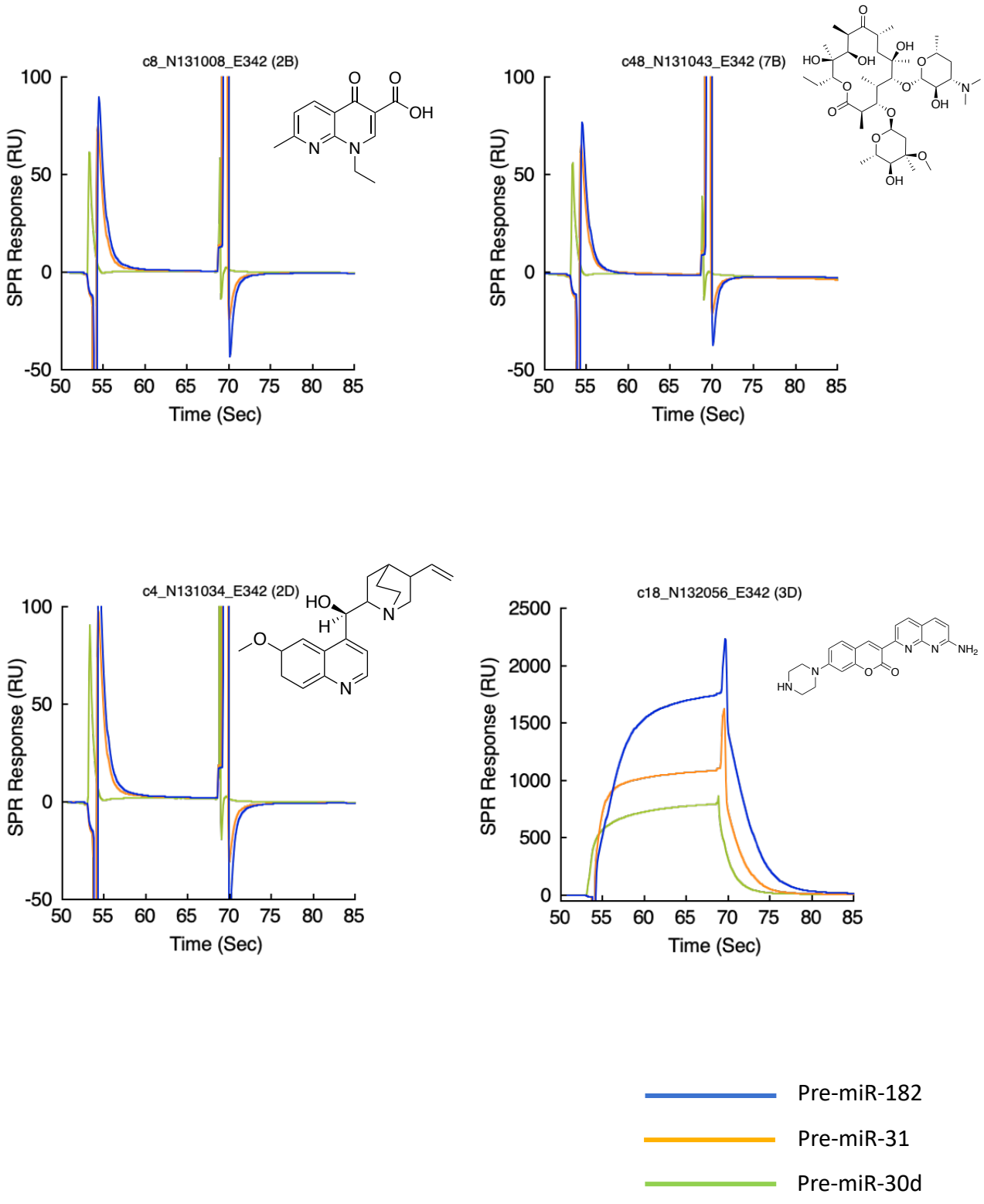
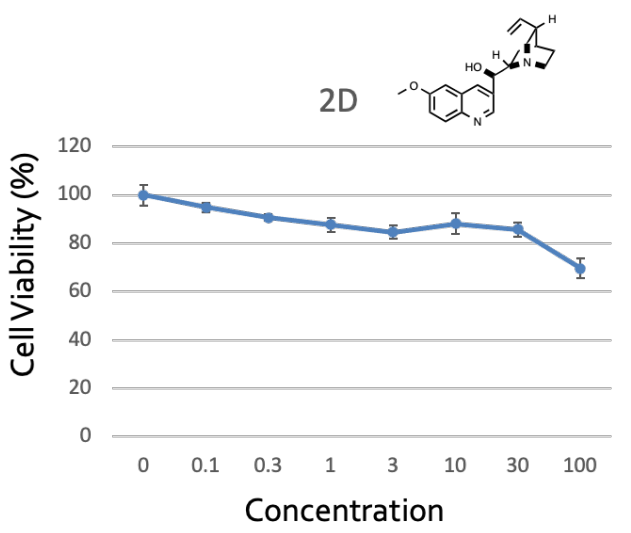


Figure 3.14 SPR binding assay of compound 2B, 7B, 2D, and 3D to the pre-miR-182/31/30d immobilized surface. Ligand was added at concentration of 50 uM. The amount of pre-miR-182/31/30d immobilized on SA chip was 1606.8, 2150, 1950.7 RU respectively

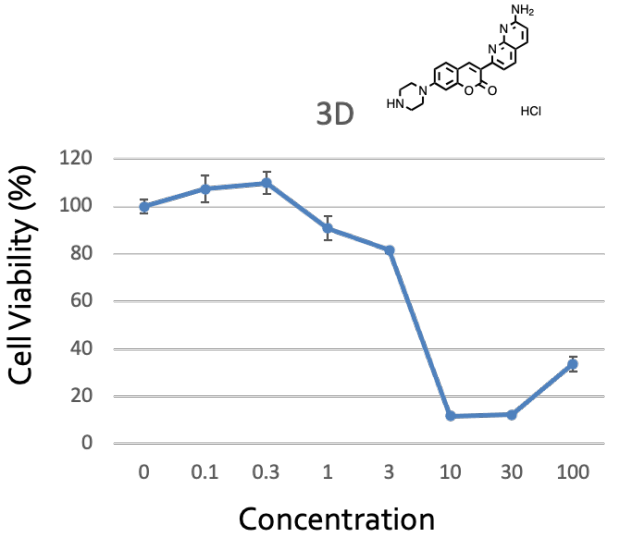
3.2.6 WST-8 Viability Assay

To assess the biological significance of eight compounds from the previous screening, we used a WST-8 viability assay to measure cell viability and proliferation. The cells were cultured and pre-incubated before adding varying concentrations of each of the eight compounds ranging from 0 to 100 uM. After another incubation, the cells' absorbance at 450 nm was measured using a microplate reader.

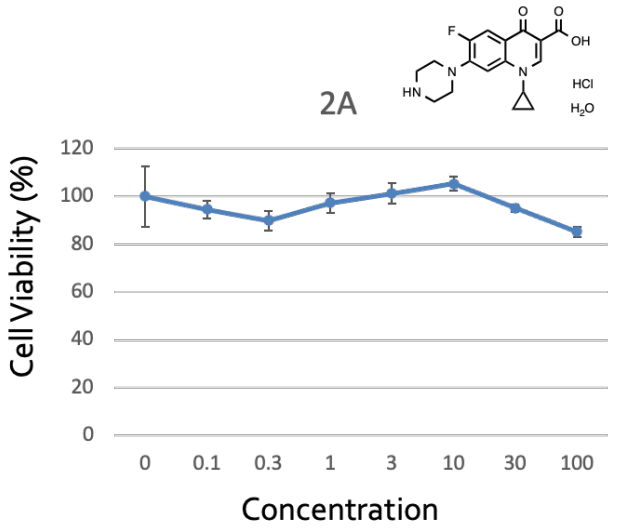
The results of this assay are depicted in Figure 3.15 and 3.16, which shows the effects of compound concentration on cell viability. The common pattern that appears in all eight figures is that the higher the concentration of the compounds, the lower the cell viability. However, among those eight compounds, compounds 3D and 4C show significant inhibition, and based on this data, we can predict that the IC_{50} for 3D is around 5 uM and 0.2 uM for 4C.



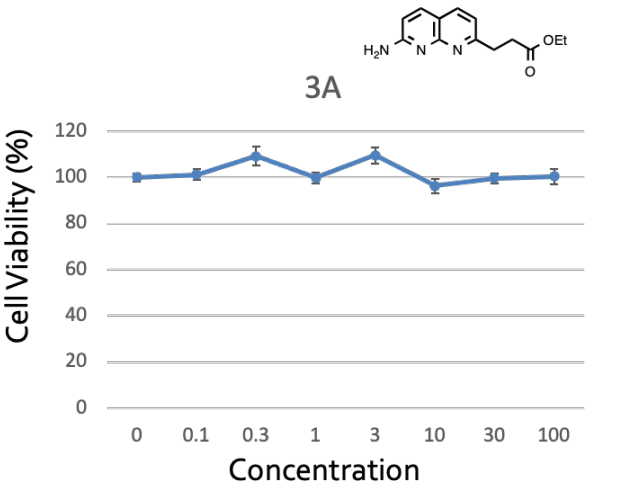
Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	94.89	90.72	87.74	84.67	88.12	85.73	69.60
Std Dev	4.34	1.98	1.41	2.97	2.58	4.24	2.81	4.00



Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	107.44	109.99	90.84	81.71	11.78	12.30	33.81
Std Dev	2.98	5.63	4.60	5.16	1.14	0.67	0.30	3.10

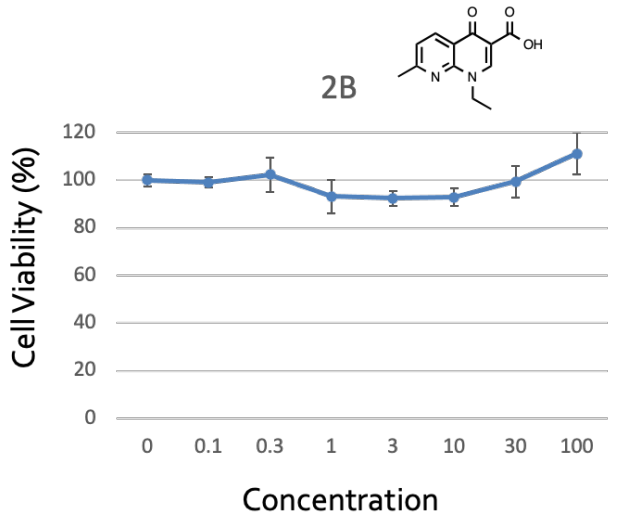


Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	94.68	89.91	97.37	101.26	105.41	95.24	85.32
Std Dev	12.68	3.67	4.05	4.00	4.33	2.87	1.50	2.21

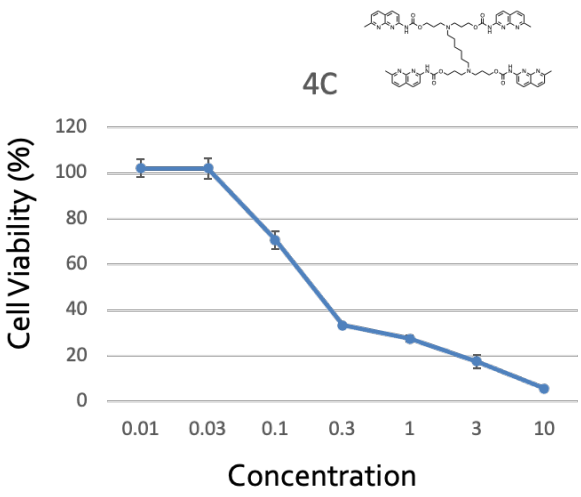


Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	101.13	109.23	99.75	109.54	96.30	99.54	100.36
Std Dev	1.77	2.38	4.06	2.47	3.49	3.08	2.07	3.38

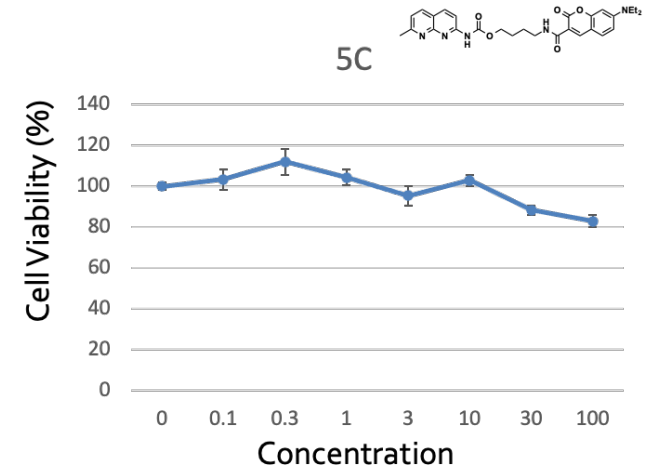
Figure 3.15 Viability assay of compound 2D, 3D, 2A and 3A



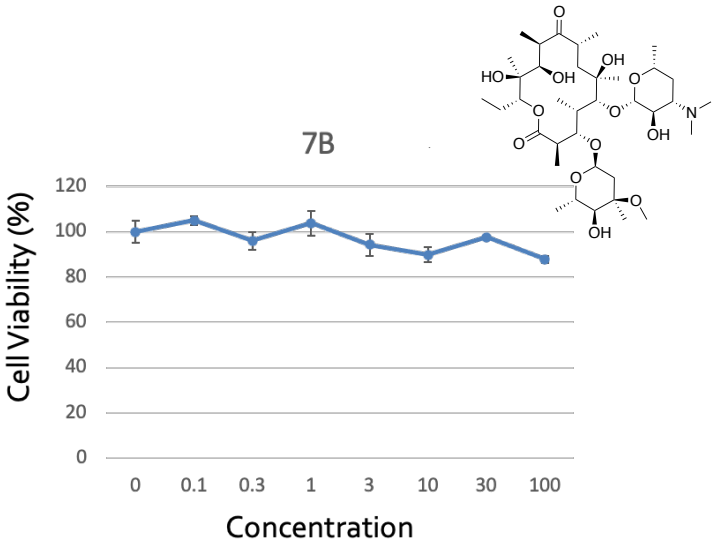
Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	99.10	102.39	93.14	92.49	92.89	99.38	111.17
Std Dev	2.60	2.17	7.29	7.16	3.12	3.55	6.72	8.78



Conc (uM)	0.01	0.03	0.1	0.3	1	3	10
Via %	102.14	102.04	70.69	33.39	27.55	17.60	5.79
Std Dev	3.87	4.59	3.75	0.34	1.34	2.92	0.40



Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	103.32	111.92	104.41	95.42	102.84	88.34	82.93
Std Dev	1.92	5.08	6.22	4.00	4.70	2.58	2.25	2.88



Conc (uM)	0	0.1	0.3	1	3	10	30	100
Via %	100.00	105.05	96.06	103.78	94.24	89.79	97.59	87.81
Std Dev	4.76	1.91	3.96	5.56	4.71	3.29	0.54	1.49

Figure 3.16 Viability assay of compound 2B, 4C, 5C and 7B

3.2.7 The Mechanism of Action

Based on the screening results, it was known that the the miRNA production was inhibited by all of the hit compounds, however, among 8 hit compounds (2A, 2B, 2D, 3A, 3D, 4C, 5C, and 7B), only 2 compounds that shows binding affinity to miRNA precursors (3D and 4C). To understand this phenomenon, we could refer to the first screening assay. From that assay (the *In vitro* dicer reaction), the reaction condition has been limited to the miRNA precursors, Dicer, and buffer. It means there are two possibilities regarding the miRNA production inhibition mechanism as shown in Figure 3.16, either by binding to the miRNA precursors and inhibit the Dicer-mediated cleavage reaction, or these compounds directly affecting the Dicer thereby inhibit the miRNA production.

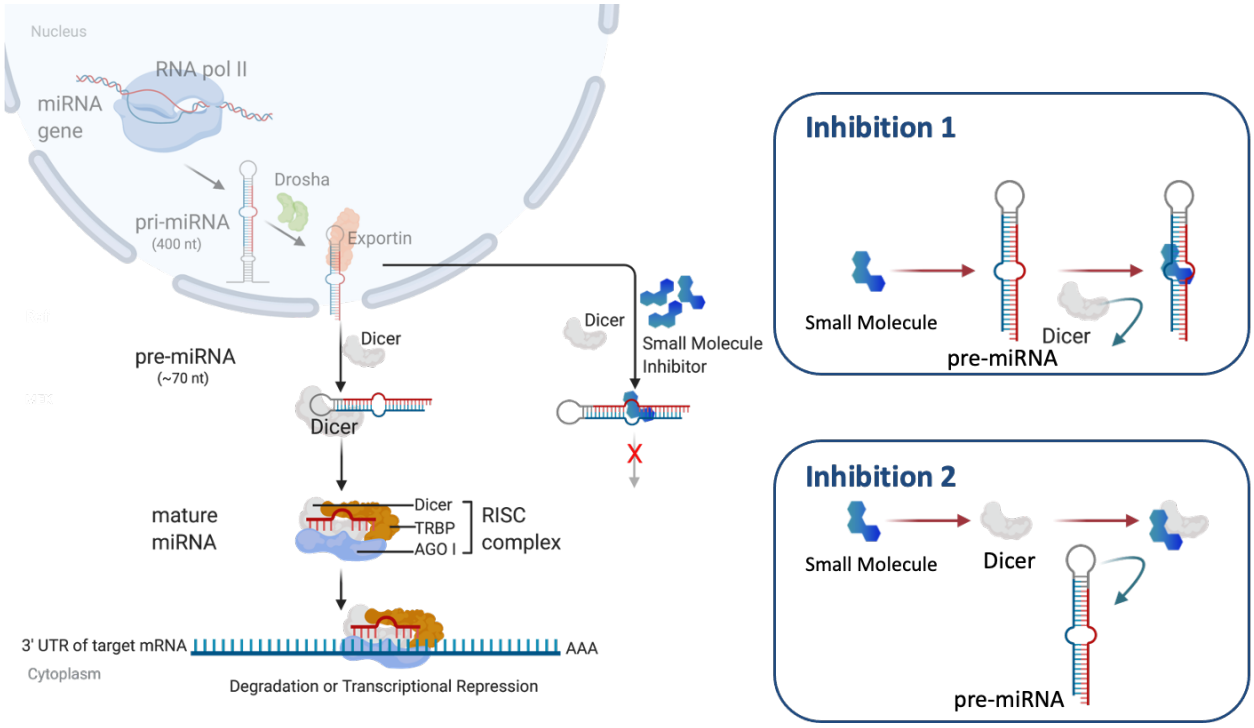


Figure 3.17 Inhibition mechanism; (Inhibition 1) small molecule bind to pre-miRNA and inhibit Dicer-mediated cleavage reaction, (Inhibition 2) small molecule affecting the Dicer, and it makes the inhibition occurred.

3.3 Conclusions

We performed a screening assay based on our understanding of miRNA biogenesis. We used the *in vitro* dicer cleavage reaction in our primary screening and we were able to found 48 compounds from the initial 67 compounds as potential inhibitors. These 48 compounds then subjected to in-cell screening as the secondary screening, and we were able to obtain 8 compounds (2A, 2B, 2D, 3A, 3D, 4C, 5C, and 7B) with promising results.

Aside from that, we conducted a similar in-cell screening for 19 excluded compounds from the primary screening to evaluate our screening criteria, and the result shows that all 19 compounds did not pass the second screening, indicating that the criteria that were used in the first screening are valid.

Additional assays were also performed to learn more about the biological significance of those eight compounds. According to the SPR analysis and viability assay, compound 3D was the most promising result as an inhibitor to miR-182 production. Compound 4C also shows similar results, however, its low IC_{50} value during viability assay makes the second screening of this compound compromised and needs to be analyzed further before using this compound on the further assay.

3.4 Methods

3.4.1 The Library Compounds and pre-microRNA targets

All chemical compounds and microRNA precursors (pre-miR-182/31/30d) belong to the Nakatani laboratory library. 67 different compounds that were used in this experiment were selected by considering the structural difference of every compound group. To make it easier to identify, the nomenclature in this experiment was used by giving letters and numbers according to the location of the compounds in the library well-plate. The re-arrangement of the 48-compounds also applied after the first screening.

3.4.2 In Vitro Dicer Cleavage Reaction

In the presence of a buffer reaction, phosphorylated precursor microRNA (0.5 uM), 50 mM $MgCl_2$, 10 mM ATP, 0.05 percent BSA, and Dicer (50 nM) were mixed. The sample from the library compounds was then added, with RNAse-free water serving as a negative control. After that, the mixture was incubated at 37^{o}C for 6 hours. The reaction was then halted by adding 10 mM EDTA. To remove non-nucleic acids, ethanol precipitation was used, followed by RNAse-free water to solute the nucleic acids.

3.4.3 In-cell Reaction

HeLa cells were cultured and expanded using standard techniques. The cells were then plated in multi-well plates and incubated in the incubator overnight (37^{o}C, 5% CO_2). At each well, the cell density is 5000 cells per 100 uL. Following incubation, HeLa cells were treated with a final concentration of the target compounds of 1 uM. Another overnight incubation was performed to ensure that the desired biological effect was achieved. Following treatment, the lysis and cDNA procedures were carried out in accordance with the kit's manufacturer's instructions.

3.4.4 WST-8 Cell Viability Assay

After dispensing 100uL of cell suspension (5000 cells/well) into a 96-well plate, a 24-hour pre-incubation period in a humidified incubator was performed. Following that, 10 uL of the substances to be tested at various concentrations was added. Another 24-hour incubation period was required to determine the effect of the substances. Then CCK-8 was added and incubated for an additional 1-4 hours. After that, using a microplate reader, determine the absorbance at 450 nM.

CHAPTER IV

REFERENCES

1. Bartel DP., 2018, Metazoan MicroRNAs. *Cell* . 173. 20-51
2. Bertel DP., 2009, MicroRNAs: target recognition and regulatory functions, *Cell*, 136, 215-233
3. O'Brien J, Hayder H, Zayed Y, Peng C., 2018, Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol.* 3, 9, 402.
4. Misiewicz-Krzeminska I, Krzeminski P, Corchete LA, et al. 2019, Factors Regulating microRNA Expression and Function in Multiple Myeloma. *Noncoding RNA*, 5, 1, 9
5. *Chen Q, Zhang H, Zhang J, Shen L, Yang J, Wang Y, Ma J, Zhuan B,* 2021*,* miR-210-3p Promotes Lung Cancer Development and Progression by Modulating USF1 and PCGF3. *Onco Targets Ther.* 14, 3687-3700
6. *Itani MM, Nassar FJ, Tfayli AH, Talhouk RS, Chamandi GK, Itani ARS, Makoukji J, Boustany RN, Hou L, Zgheib NK, Nasr RR* , 2021, A Signature of Four Circulating microRNAs as Potential Biomarkers for Diagnosing Early-Stage Breast Cancer, *Int J Mol Sci, 22, 11*
7. *Xu H, Ding Y, Yang X,* 2021*,* Overexpression of Long Noncoding RNA H19 Downregulates miR-140-5p and Activates PI3K/AKT Signaling Pathway to Promote Invasion, Migration and Epithelial-Mesenchymal Transition of Ovarian Cancer Cells. *Biomed Res Int. 2021, 6619730.*
8. *Zhong S, Golpon H, Zardo P, Borlak J* , 2021, miRNAs in lung cancer. A systematic review identifies predictive and prognostic miRNA candidates for precision medicine in lung cancer. *Transl Res. , 230, 164-196.*
9. Dias N, Stein CA, 2002, Antisense oligonucleotides: basic concepts and mechanism. *Mol Cancer Ther*.,1,5, 347-355
10. Krutzfeldt J, Rajewsky N, Braich R, et al., 2005, Silencing of microRNA *In vivo* with Antagomirs, *Nature*, 438, 7068, 685-689
11. Petersen M, Nielsen CB, Nielsen KE, Jensen GA, Bondensgaard K, Singh SK, Rajwanshi VK, Koshkin AA, Dahl BM, Wengel J, Jacobsen JP., 2000, The conformations of locked nucleic acids (LNA). J Mol Recognit. 13(1):44-53.

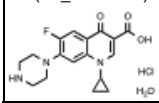
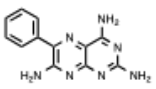
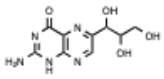
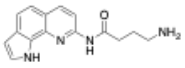
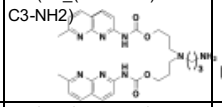
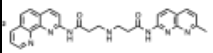
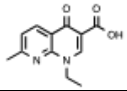
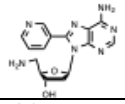
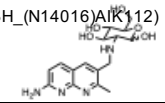
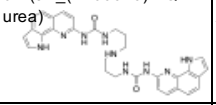
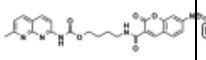
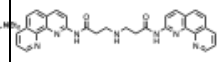
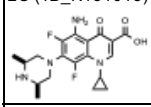
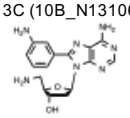
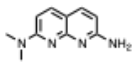
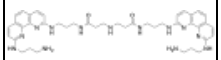
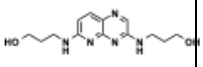
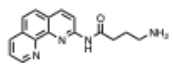
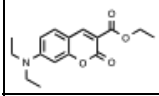
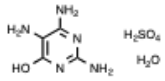
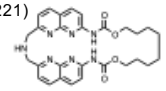
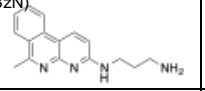
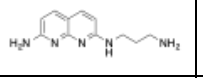
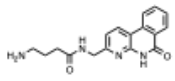
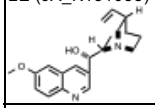
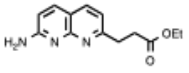
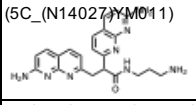
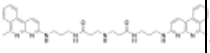
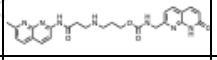
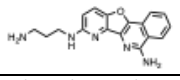
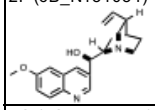
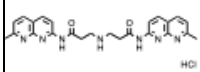
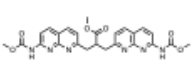
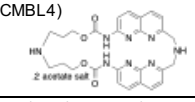
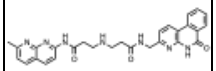
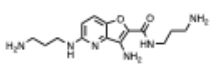
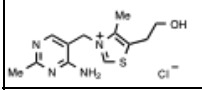
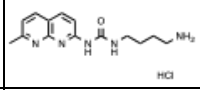
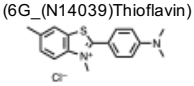
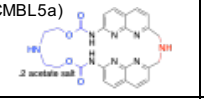
12. Wengel J, Vester B, Lundberg LB, Douthwaite S, Sørensen MD, Babu BR, Gait MJ, Arzumanov A, Petersen M, Nielsen JT. , 2003, LNA and alpha-L-LNA: towards therapeutic applications. Nucleosides Nucleotides Nucleic Acids. 22(5-8):601-4.
13. Zhao Y, Dai Z, Liang Y, Yin M, Ma K, He M, Ouyang H, Teng CB. 2014, Sequence-specific inhibition of microRNA via CRISPR/CRISPRi system. *Sci Rep.,* 3;4:3943.
14. Xiao A, Wang Z, Hu Y, Wu Y, Luo Z, Yang Z, Zu Y, Li W, Huang P, Tong X, Zhu Z, Lin S, Zhang B., 2013, Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res*. 41(14):e141.
15. Galbraith WM, Hobson WC, Giclas PC, Schechter PJ, Agrawal S. , 1994, Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res Dev*. ,4(3):201-6.
16. Li Z, Yang CS, Nakashima K, Rana TM. Small RNA-mediated regulation of iPS cell generation. EMBO J. 2011 Mar 2;30(5):823-34.
17. Xiao-Jie L, Hui-Ying X, Zun-Ping K, Jin-Lian C, Li-Juan J., . 2015, CRISPR-Cas9: a new and promising player in gene therapy. J Med Genet, 52(5):289-96.
18. Nathan P. Coussens, John C. Braisted, Tyler Peryea, G. Sitta Sittampalam, Anton Simeonov , and Matthew D. Hall, 2017, Small-Molecule Screens: A Gateway to Cancer Therapeutics, *Pharmacological Reviews*,1, 69 (4) 479-496;
19. Acker MG and Auld DS, 2014, Considerations for the design and reporting of enzyme assays in high throughput screening applications, *Perspect Sci*, 1, 56-73
20. Haslam C, Hellicar J, Dunn A, Fuetterer A, Hardy N, Marshall P, Paape R, Pemberton M, Resemannand A, Leveridge M. , 2016, The Evolution of MALDI-TOF Mass Spectrometry toward Ultra-High-Throughput Screening: 1536-Well Format and Beyond. *J Biomol Screen*. 21(2):176-86.
21. Shoemaker RH., 2006, The NCI60 human tumor cell line anticancer drug screen. *Nat Rev Cancer*. 6(10):813-23.
22. Susan L. Holbeck, Jerry M. Collins and James H. Doroshow, 2010, Analysis of Food and Drug Administration–Approved Anticancer Agents in the NCI60 Panel of Human Tumor Cell Lines, 9, 1451-1460
23. Eder J, Sedrani R, Wiesmann C., 2014, The discovery of first-in-class drugs: origins and evolution. Nat Rev Drug Discov., 13(8):577-87.

24. Zhao Y, Mu X, Du G., 2016, Microtubule-stabilizing agents: New drug discovery and cancer therapy. Pharmacol Ther. , 162:134-43.
25. Moffat JG, Rudolph J, Bailey D., 2014, Phenotypic screening in cancer drug discovery past, present, and future. *Nat Rev Drug Discov*. 13(8):588-602
26. Sponholtz, T.R., et al.,2017, *Cancer Causes Control*, 28 (6), 579–588
27. Gang Du , Wenqiang Zhang, Zhen Zhang, et al, 2019, HTLV-1-associated genes as potential biomarkers for endometrial cancer, 18, 1, 699-705
28. Siegel, R.L., Miller, K.D., and Jemal A., 2017. *C.A. Cancer J. Clin. ,* 67(1), 7–30.
29. Shaily Arora, Sanjeev Balasubramaniam, Wei Zhang, Lijun Zhang, Rajeshwari Sridhara, Dianne Spillman, Jaigi P. Mathai, Bradley Scott, Sarah J. Golding, Michael Coory, Richard Pazdur and Julia A. Beaver, 2020, FDA Approval Summary: Pembrolizumab plus Lenvatinib for Endometrial Carcinoma, a Collaborative International Review under Project Orbis, *Clin Cancer Res*, 26, 19, 5062-5067
30. Favier A, Rocher G, Larsen AK, Delangle R, Uzan C, Sabbah M, Castela M, Duval A, Mehats C, Canlorbe G., 2021, MicroRNA as Epigenetic Modifiers in Endometrial Cancer: A Systematic Review. Cancers (Basel)., 13, 5, 1137.
31. Gunson RN, Collins TC, Carman WF. Practical experience of high throughput real-time PCR in the routine diagnostic virology setting. *J Clin Virol*. 2006;35(4):355-367.
32. Kramer MF., 2011, Stem-loop RT-qPCR for miRNAs. *Curr Protoc Mol Biol*, 10-15.10
33. Nakatani, K. 2009, Recognition of Mismatched base pairs in DNA. *Bull. Chem. Soc. Jpn*, 82, 1055-1069
34. K. Nakatani, S. Sando, I. Saito, 2001, Nat. Biotechnol., 19, 51.
35. K. Nakatani, S. Sando, H. Kumasawa, J. Kikuchi, I. Saito, 2001, J. Am. Chem. Soc., 123, 12650.
36. K. Nakatani, S. Sando, I. Saito, 2001, Bioorg. Med. Chem., 9, 2381.
37. S. Hagihara, H. Kumasawa, Y. Goto, G. Hayashi, A. Kobori, I. Saito, K. Nakatani, 2004, Nucleic Acids Res., 32, 278
38. A. Kobori, S. Horie, H. Suda, I. Saito, K. Nakatani, 2004, J. Am. Chem. Soc. 126, 557.
39. H. Suda, A. Kobori, J. Zhang, G. Hayashi, K. Nakatani, 2005, *N*,*N*'-Bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine stabilized a single pyrimidine bulge in duplex DNA, Bioorg. Med. Chem 13, 14, 4507-4512

40. A. Murata, T. Otabe, J. Zhang, K. Nakatani, 2016, BzDANP, a Small-Molecule Modulator of pre-miR-29a Maturation by Dicer, *ACS Chem Biol*, 11, 10, 2790-2796
41. Otabe T, Nagano K, Kawai G, Murata A, Nakatani K., 2019, Inhibition of pre-miRNA-136 processing by Dicer with small molecule BzDANP suggested the formation of ternary complex of pre-miR-136-BzDANP-Dicer. *Bioorg Med Chem*. 15, 27, 10, 2140-2148.

SUPPLEMENTARY DATA

Well Plate Rearrangement

2A (2B_N131002) 	3A (8D_N131052) 	4H (11H_N132080) 	6G (2G_(N160007) PQA-C3-amide) 	7G (6C_(N160035) NCD-C3-NH2) 	9F (7G_(N14247)ljx224) 
2B (2H_N131008) 	3B (9H_N131064) 	5B (3H_(N14016)AIK112) 	6H (3B_(N160010) PQAD-urea) 	7H (7F_(N160046) AIK216) 	9G (8A_(N14249)ljx238) 
2C (4B_N131018) 	3C (10B_N131066) 	5C (4B_(N14018)SA029) 	7A (3C_(N160011) DDAP) 	8A (7H_(N160048) AIK248) 	9H (8B_(N14250)ljx223) 
2D (4G_N131023) 	3D (11H_N131080) 	5D (4H_(N14024)SM-221) 	7B (4B_(N160018) Am-BzN) 	8B (8D_(N160052) ih 039) 	10C (9B_(N14258)ljx188) 
2E (6A_N131033) 	3E (2A_N132001) 	5E (5C_(N14027)YM011) 	7C (4C_(N160019) Am-BzND) 	9B (7B_(N14242)ljx249) 	10E (9H_(N14264)ljx372) 
2F (6B_N131034) 	3H (4H_N132024) 	5F (5E_(N14029)NN146) 	7D (4G_(N160023) CMBL4) 	9C (7C_(N14243)BzNA) 	10F (10A_(N14265)ljx380) 
2G (6G_N131039) 	4B (5C_N132027) 	5H (6G_(N14039)Thioflavin) 	7E (5A_(N160025) CMBL5a) 	9D (7D_(N14244)ljx253)	10G (10B_(N14266)ljx344)
2H (7C_N131043)	4D (8H_N132056)	6D (10E_(N14069)NCT6)	7F (5H_(N160032) NCD-C4-SH)	9E (7E_(N14245)ljx107)	10H (10D_(N14268)ljx358)

S1 Well plate rearrangement , this arrangement used for 48 compound In-Cell screening, SPR analysis, and viability assay

Sample	has-miR-182 (CT value)		dCT		2^-dCT		Threshold <0.5		R
	I	II	I	II	I	II	I	II	
2A	20.967	20.554	3.558	3.144	0.085	0.113	1	1	OK
2B	20.900	20.571	3.491	3.162	0.089	0.112	1	1	OK
2C	16.413	16.126	-0.996	-1.283	1.995	2.434	0	0	-
2D	16.614	16.456	-0.795	-0.954	1.735	1.937	0	0	-
2E	20.359	20.246	2.950	2.837	0.129	0.140	1	1	OK
2F	16.965	16.861	-0.444	-0.548	1.360	1.462	0	0	-
2G	16.140	16.317	-1.269	-1.092	2.410	2.131	0	0	-
2H	20.438	20.484	3.029	3.075	0.123	0.119	1	1	OK
3A	20.133	20.808	2.724	3.399	0.151	0.095	1	1	OK
3B	21.252	21.725	3.843	4.316	0.070	0.050	1	1	OK
3C	16.153	16.974	-1.257	-0.435	2.389	1.352	0	0	-
3D	15.838	16.146	-1.572	-1.263	2.972	2.400	0	0	-
3E	20.776	20.952	3.367	3.543	0.097	0.086	1	1	OK
3F	15.944	16.240	-1.465	-1.169	2.761	2.248	0	0	-
3G	16.943	16.962	-0.466	-0.447	1.381	1.363	0	0	-
3H	22.494	22.441	5.085	5.032	0.029	0.031	1	1	OK
4A	16.740	17.148	-0.669	-0.261	1.590	1.198	0	0	-
4B	16.091	16.457	-1.318	-0.952	2.493	1.935	0	0	-
4C	16.922	17.267	-0.487	-0.142	1.402	1.104	0	0	-
4D	22.016	22.071	4.607	4.662	0.041	0.040	1	1	OK
4E	16.308	16.554	-1.101	-0.855	2.145	1.808	0	0	-
4F	16.102	16.149	-1.307	-1.260	2.475	2.395	0	0	-
4G	16.819	16.714	-0.591	-0.695	1.506	1.619	0	0	-
4H	18.854	18.835	1.444	1.426	0.367	0.372	1	1	OK
5A	16.063	16.719	-1.346	-0.691	2.543	1.614	0	0	-
5B	20.596	21.048	3.187	3.638	0.110	0.080	1	1	OK
5C	20.689	21.183	3.279	3.773	0.103	0.073	1	1	OK
5D	20.495	20.473	3.086	3.064	0.118	0.120	1	1	OK
5E	16.500	16.400	-0.909	-1.009	1.878	2.013	0	0	-
5F	22.066	21.932	4.657	4.523	0.040	0.044	1	1	OK
5G	16.308	16.252	-1.101	-1.157	2.145	2.230	0	0	-
5H	22.123	22.000	4.714	4.591	0.038	0.041	1	1	OK
6A	15.436	16.143	-1.973	-1.266	3.926	2.405	0	0	-
6B	16.774	17.817	-0.635	0.408	1.553	0.754	0	0	-
6C	16.488	17.383	-0.921	-0.026	1.893	1.018	0	0	-
6D	23.421	24.057	6.012	6.648	0.015	0.010	1	1	OK
6E	16.539	16.712	-0.870	-0.697	1.828	1.621	0	0	-
6F	16.158	16.786	-1.251	-0.623	2.380	1.540	0	0	-
6G	16.958	17.383	-0.451	-0.027	1.367	1.019	0	0	-
6H	17.948	18.259	0.538	0.850	0.689	0.555	0	0	-
7A	22.986	22.429	5.576	5.020	0.021	0.031	1	1	OK
7B	19.617	20.033	2.207	2.624	0.217	0.162	1	1	OK
7C	24.398	24.750	6.989	7.341	0.008	0.006	1	1	OK
7D	26.438	26.395	9.029	8.985	0.002	0.002	1	1	OK
7E	21.963	22.541	4.554	5.132	0.043	0.029	1	1	OK
7F	22.584	22.961	5.175	5.552	0.028	0.021	1	1	OK
7G	19.315	19.964	1.906	2.555	0.267	0.170	1	1	OK
7H	24.096	24.376	6.686	6.967	0.010	0.008	1	1	OK
8A	17.083	17.861	-0.326	0.452	1.254	0.731	0	0	-
8B	15.972	16.623	-1.437	-0.786	2.707	1.724	0	0	-
8C	17.682	18.644	0.273	1.235	0.827	0.425	0	1	-
9A	17.210	17.690	-0.200	0.280	1.148	0.823	0	0	-
9B	16.660	16.498	-0.749	-0.911	1.681	1.880	0	0	-
9C	17.635	18.068	0.226	0.659	0.855	0.633	0	0	-
9D	16.245	17.194	-1.164	-0.215	2.241	1.161	0	0	-
9E	21.765	22.215	4.355	4.806	0.049	0.036	1	1	OK
9F	16.396	16.811	-1.013	-0.598	2.019	1.514	0	0	-
9G	18.118	18.347	0.708	0.938	0.612	0.522	0	0	-
9H	17.034	17.119	-0.375	-0.290	1.297	1.223	0	0	-
10A	16.782	16.640	-0.627	-0.769	1.545	1.704	0	0	-
10B	17.566	17.681	0.157	0.271	0.897	0.829	0	0	-
10C	21.061	20.977	3.652	3.568	0.080	0.084	1	1	OK
10D	17.785	17.729	0.376	0.320	0.771	0.801	0	0	-
10E	18.471	18.423	1.061	1.014	0.479	0.495	1	1	OK
10F	17.587	17.703	0.178	0.293	0.884	0.816	0	0	-
10G	16.964	17.187	-0.445	-0.222	1.361	1.167	0	0	-
10H	17.413	17.448	0.004	0.039	0.997	0.974	0	0	-
Reference	17.409								

S2 *In vitro* Dicer reaction detail calculation for hsa-miR-182

Sample	has-miR-31 (CT value)		dCT		2^-dCT		Threshold <0.5		R
	I	II	I	II	I	II	I	II	
2A	15.295	14.664	-0.758	-1.390	1.691	2.620	0	0	-
2B	15.775	15.386	-0.278	-0.667	1.212	1.588	0	0	-
2C	16.301	16.069	0.248	0.015	0.842	0.989	0	0	-
2D	15.596	15.373	-0.457	-0.680	1.373	1.602	0	0	-
2E	16.887	16.431	0.833	0.378	0.561	0.770	0	0	-
2F	17.223	17.270	1.170	1.216	0.444	0.430	1	1	OK
2G	17.367	17.361	1.313	1.308	0.402	0.404	1	1	OK
2H	17.345	17.368	1.292	1.314	0.409	0.402	1	1	OK
3A	18.994	18.526	2.941	2.472	0.130	0.180	1	1	OK
3B	17.907	17.707	1.853	1.654	0.277	0.318	1	1	OK
3C	17.695	17.198	1.642	1.144	0.320	0.452	1	1	OK
3D	18.603	18.340	2.550	2.287	0.171	0.205	1	1	OK
3E	16.841	16.731	0.788	0.678	0.579	0.625	0	0	-
3F	16.216	15.853	0.163	-0.200	0.893	1.149	0	0	-
3G	16.560	16.239	0.507	0.186	0.704	0.879	0	0	-
3H	16.409	16.294	0.356	0.241	0.781	0.846	0	0	-
4A	16.184	15.890	0.131	-0.163	0.913	1.120	0	0	-
4B	17.782	17.509	1.729	1.456	0.302	0.365	1	1	OK
4C	16.778	16.678	0.724	0.624	0.605	0.649	0	0	-
4D	19.425	19.258	3.371	3.204	0.097	0.108	1	1	OK
4E	16.188	16.009	0.135	-0.045	0.911	1.031	0	0	-
4F	16.550	16.550	0.497	0.497	0.709	0.709	0	0	-
4G	16.357	16.523	0.304	0.470	0.810	0.722	0	0	-
4H	19.145	19.328	3.091	3.275	0.117	0.103	1	1	OK
5A	16.758	16.519	0.704	0.466	0.614	0.724	0	0	-
5B	16.663	16.149	0.609	0.096	0.655	0.936	0	0	-
5C	16.964	16.772	0.911	0.719	0.532	0.607	0	0	-
5D	16.755	16.444	0.702	0.391	0.615	0.763	0	0	-
5E	16.103	16.049	0.050	-0.005	0.966	1.003	0	0	-
5F	16.065	15.879	0.012	-0.175	0.992	1.129	0	0	-
5G	15.996	15.716	-0.057	-0.337	1.040	1.263	0	0	-
5H	15.096	14.777	-0.957	-1.276	1.942	2.422	0	0	-
6A	15.897	15.689	-0.157	-0.364	1.115	1.287	0	0	-
6B	16.357	16.302	0.304	0.249	0.810	0.842	0	0	-
6C	16.745	16.631	0.692	0.577	0.619	0.670	0	0	-
6D	20.875	20.556	4.822	4.503	0.035	0.044	1	1	OK
6E	15.414	15.410	-0.639	-0.643	1.557	1.562	0	0	-
6F	16.080	16.247	0.026	0.193	0.982	0.875	0	0	-
6G	17.685	17.540	1.632	1.487	0.323	0.357	1	1	OK
6H	22.738	22.976	6.685	6.923	0.010	0.008	1	1	OK
7A	19.979	19.712	3.926	3.658	0.066	0.079	1	1	OK
7B	16.809	16.687	0.755	0.634	0.592	0.644	0	0	-
7C	20.206	19.769	4.153	3.716	0.056	0.076	1	1	OK
7D	24.883	24.534	8.830	8.481	0.002	0.003	1	1	OK
7E	19.597	19.508	3.544	3.454	0.086	0.091	1	1	OK
7F	22.339	22.534	6.286	6.481	0.013	0.011	1	1	OK
7G	19.407	19.319	3.353	3.265	0.098	0.104	1	1	OK
7H	15.908	15.934	-0.145	-0.119	1.106	1.086	0	0	-
8A	21.585	21.387	5.532	5.334	0.022	0.025	1	1	OK
8B	15.136	15.071	-0.917	-0.982	1.888	1.975	0	0	-
8C	13.753	13.855	-2.300	-2.198	4.924	4.588	0	0	-
9A	14.995	14.727	-1.058	-1.326	2.082	2.508	0	0	-
9B	17.979	17.846	1.926	1.793	0.263	0.289	1	1	OK
9C	20.919	20.884	4.866	4.831	0.034	0.035	1	1	OK
9D	21.625	21.392	5.572	5.339	0.021	0.025	1	1	OK
9E	22.151	22.021	6.098	5.967	0.015	0.016	1	1	OK
9F	17.755	17.440	1.702	1.387	0.307	0.382	1	1	OK
9G	35.006	35.217	18.953	19.164	0.000	0.000	1	1	OK
9H	23.997	23.881	7.943	7.828	0.004	0.004	1	1	OK
10A	16.557	16.341	0.504	0.288	0.705	0.819	0	0	-
10B	16.034	15.694	-0.020	-0.359	1.014	1.283	0	0	-
10C	20.795	20.586	4.742	4.532	0.037	0.043	1	1	OK
10D	15.751	15.574	-0.302	-0.479	1.233	1.394	0	0	-
10E	19.609	19.448	3.555	3.394	0.085	0.095	1	1	OK
10F	24.209	24.154	8.156	8.101	0.004	0.004	1	1	OK
10G	16.289	16.197	0.236	0.144	0.849	0.905	0	0	-
10H	24.786	24.592	8.733	8.539	0.002	0.003	1	1	OK
Reference	16.053								

S3 *In vitro* Dicer reaction detail calculation for hsa-miR-31

Sample	hsa-miR-30d (CT value)		dCT		2^-dCT		Threshold <0.5		R
	I	II	I	II	I	II	I	II	
2A	16.119	16.099	1.683	1.663	0.311	0.316	1	1	OK
2B	15.342	15.393	0.906	0.957	0.533	0.515	0	0	-
2C	15.956	15.953	1.520	1.517	0.349	0.349	1	1	OK
2D	16.281	16.199	1.845	1.763	0.278	0.295	1	1	OK
2E	14.843	14.807	0.407	0.371	0.754	0.773	0	0	-
2F	14.607	14.538	0.171	0.102	0.888	0.932	0	0	-
2G	15.714	15.709	1.278	1.273	0.412	0.414	1	1	OK
2H	15.927	15.978	1.491	1.542	0.356	0.343	1	1	OK
3A	18.211	18.168	3.776	3.732	0.073	0.075	1	1	OK
3B	17.709	17.613	3.273	3.177	0.103	0.111	1	1	OK
3C	16.028	15.960	1.593	1.524	0.332	0.348	1	1	OK
3D	17.012	16.931	2.576	2.496	0.168	0.177	1	1	OK
3E	14.540	14.374	0.104	-0.062	0.930	1.044	0	0	-
3F	14.597	14.560	0.161	0.124	0.895	0.918	0	0	-
3G	15.076	14.933	0.641	0.497	0.641	0.709	0	0	-
3H	15.966	15.736	1.531	1.301	0.346	0.406	1	1	OK
4A	13.268	13.226	-1.168	-1.210	2.247	2.313	0	0	-
4B	13.739	13.604	-0.697	-0.831	1.621	1.780	0	0	-
4C	14.857	14.675	0.421	0.239	0.747	0.847	0	0	-
4D	15.444	15.368	1.009	0.932	0.497	0.524	1	0	-
4E	13.995	13.906	-0.440	-0.530	1.357	1.444	0	0	-
4F	13.558	13.410	-0.878	-1.026	1.837	2.036	0	0	-
4G	14.236	14.118	-0.200	-0.318	1.148	1.247	0	0	-
4H	14.778	14.629	0.342	0.193	0.789	0.875	0	0	-
5A	13.822	13.768	-0.614	-0.668	1.530	1.588	0	0	-
5B	13.964	13.787	-0.471	-0.649	1.386	1.568	0	0	-
5C	13.951	13.786	-0.485	-0.650	1.400	1.569	0	0	-
5D	16.310	16.231	1.874	1.795	0.273	0.288	1	1	OK
5E	15.535	15.498	1.100	1.062	0.467	0.479	1	1	OK
5F	14.681	14.615	0.245	0.179	0.844	0.884	0	0	-
5G	15.053	14.882	0.618	0.446	0.652	0.734	0	0	-
5H	15.582	15.378	1.146	0.942	0.452	0.521	1	0	-
6A	14.212	14.371	-0.224	-0.065	1.168	1.046	0	0	-
6B	14.971	15.073	0.535	0.638	0.690	0.643	0	0	-
6C	14.440	14.501	0.004	0.065	0.997	0.956	0	0	-
6D	13.529	13.621	-0.907	-0.815	1.876	1.759	0	0	-
6E	13.957	13.850	-0.478	-0.586	1.393	1.501	0	0	-
6F	14.037	14.064	-0.399	-0.372	1.318	1.294	0	0	-
6G	14.676	14.832	0.241	0.397	0.846	0.760	0	0	-
6H	17.277	17.367	2.841	2.932	0.140	0.131	1	1	OK
7A	18.869	18.787	4.433	4.351	0.046	0.049	1	1	OK
7B	14.476	14.409	0.040	-0.027	0.973	1.019	0	0	-
7C	19.365	19.372	4.929	4.936	0.033	0.033	1	1	OK
7D	19.639	19.637	5.203	5.201	0.027	0.027	1	1	OK
7E	15.990	15.823	1.554	1.387	0.340	0.382	1	1	OK
7F	17.933	17.837	3.498	3.401	0.089	0.095	1	1	OK
7G	15.260	15.242	0.824	0.806	0.565	0.572	0	0	-
7H	16.791	16.621	2.355	2.185	0.195	0.220	1	1	OK
8A	14.628	14.518	0.193	0.082	0.875	0.945	0	0	-
8B	15.901	15.776	1.465	1.340	0.362	0.395	1	1	OK
8C	13.991	13.990	-0.444	-0.446	1.361	1.362	0	0	-
9A	13.627	13.432	-0.809	-1.004	1.752	2.005	0	0	-
9B	13.970	13.957	-0.466	-0.479	1.381	1.394	0	0	-
9C	12.953	12.914	-1.482	-1.522	2.794	2.871	0	0	-
9D	13.908	13.898	-0.528	-0.538	1.442	1.451	0	0	-
9E	14.314	14.315	-0.121	-0.121	1.088	1.088	0	0	-
9F	13.760	13.675	-0.676	-0.761	1.597	1.695	0	0	-
9G	13.556	13.504	-0.880	-0.932	1.840	1.908	0	0	-
9H	18.375	18.328	3.939	3.892	0.065	0.067	1	1	OK
10A	12.831	12.665	-1.605	-1.771	3.042	3.413	0	0	-
10B	14.277	14.173	-0.159	-0.263	1.117	1.200	0	0	-
10C	13.769	13.541	-0.667	-0.894	1.587	1.859	0	0	-
10D	13.731	13.506	-0.705	-0.930	1.630	1.905	0	0	-
10E	13.895	13.871	-0.541	-0.565	1.455	1.480	0	0	-
10F	13.913	13.802	-0.523	-0.634	1.436	1.551	0	0	-
10G	15.696	15.445	1.260	1.009	0.417	0.497	1	1	OK
10H	15.260	15.076	0.824	0.641	0.565	0.641	0	0	-
Reference	14.436								

S4 *In vitro* Dicer reaction detail calculation for hsa-miR-30d

Sample	U6			hsa-miR-182			Average	dCT 182-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	I	II	III	I	II	III	I	II	III	I	II	III	
2A	13.656	12.798	13.272	25.345	25.108	25.262	13.242	25.238	11.689	12.309	11.990	0.369	1.110	1.356	0.774	0.463	0.391	0	1	1	OK
2B	13.685	13.517	13.729	25.963	24.989	25.193	13.644	25.382	12.278	11.472	11.464	0.959	0.272	0.831	0.514	0.828	0.562	0	0	0	-
2C	13.212	13.819	14.066	25.314	25.048	25.045	13.699	25.136	12.102	12.229	10.979	0.783	0.029	0.346	0.581	0.980	0.787	0	0	0	-
2D	12.777	12.297	13.451	25.796	25.246	25.260	12.842	25.434	13.018	12.949	11.809	1.699	1.749	1.176	0.308	0.297	0.443	0	1	1	OK
2E	13.040	13.680	13.571	25.272	24.976	24.461	13.431	24.903	12.231	12.296	10.890	0.912	0.096	0.256	0.532	0.935	0.837	0	0	0	-
2F	13.849	12.478	13.550	25.260	24.741	24.967	13.292	24.989	11.411	12.262	11.418	0.092	1.063	0.784	0.938	0.479	0.581	0	1	0	-
2G	14.007	13.920	13.483	24.973	24.730	24.837	13.803	24.846	10.965	10.809	11.354	-0.354	-0.390	0.720	1.278	1.311	0.607	0	0	0	-
2H	13.175	13.311	13.875	24.915	24.862	25.024	13.454	24.934	11.740	11.550	11.149	0.421	0.351	0.515	0.747	0.784	0.700	0	0	0	-
3A	13.124	12.947	12.899	25.480	25.138	24.968	12.990	25.195	12.355	12.191	12.069	1.036	0.991	1.435	0.488	0.503	0.370	1	0	1	OK
3B	14.250	14.032	14.224	25.135	25.300	24.677	14.169	25.037	10.885	11.268	10.453	-0.435	0.068	-0.181	1.352	0.954	1.134	0	0	0	-
3C	13.469	13.456	13.932	25.477	25.084	24.323	13.619	24.962	12.009	11.627	10.391	0.689	0.428	-0.242	0.620	0.743	1.183	0	0	0	-
3D	13.160	12.202	13.578	25.489	25.388	25.484	12.980	25.454	12.330	13.186	11.905	1.010	1.986	1.272	0.496	0.252	0.414	1	1	1	OK
3E	13.160	13.227	13.737	25.584	24.992	24.793	13.375	25.123	12.424	11.766	11.056	1.105	0.566	0.422	0.465	0.676	0.746	0	0	0	-
3F	13.881	12.711	14.667	25.381	24.871	24.823	13.753	25.025	11.500	12.161	10.156	0.181	0.961	-0.478	0.882	0.514	1.392	0	0	0	-
3G	13.061	13.285	14.196	25.392	24.970	24.441	13.514	24.934	12.331	11.685	10.245	1.011	0.485	-0.389	0.496	0.715	1.309	0	1	0	-
3H	13.312	13.157	13.875	24.908	25.603	24.785	13.448	25.099	11.596	12.445	10.910	0.277	1.246	0.276	0.825	0.422	0.826	0	0	0	-
4A	13.546	13.598	13.624	25.448	24.979	24.390	13.589	24.939	11.903	11.381	10.767	0.584	0.181	0.133	0.667	0.882	0.912	0	0	0	-
4B	14.024	13.883	13.925	25.326	25.229	24.765	13.944	25.107	11.303	11.347	10.840	-0.017	0.147	0.207	1.012	0.903	0.867	0	0	0	-
4C	12.975	12.894	12.776	25.305	25.049	24.329	12.882	24.895	12.330	12.156	11.554	1.011	0.956	0.920	0.496	0.516	0.529	0	1	0	-
4D	14.252	13.858	13.813	25.519	25.925	24.704	13.975	25.382	11.266	12.067	10.891	-0.053	0.867	0.257	1.037	0.548	0.837	0	0	0	-
4E	14.070	13.183	13.687	25.493	25.108	24.470	13.647	25.024	11.423	11.924	10.783	0.104	0.725	0.149	0.930	0.605	0.902	0	0	0	-
4F	13.528	13.163	13.455	25.533	25.230	24.897	13.382	25.220	12.005	12.067	11.441	0.686	0.867	0.807	0.622	0.548	0.571	0	0	0	-
4G	14.286	13.824	14.022	25.495	25.381	24.442	14.044	25.106	11.209	11.557	10.420	-0.110	0.358	-0.214	1.079	0.780	1.160	0	0	0	-
4H	12.958	13.353	12.901	25.135	24.860	24.351	13.071	24.782	12.178	11.507	11.450	0.858	0.307	0.816	0.552	0.808	0.568	0	0	0	-
5A	13.886	13.692	13.583	25.206	25.104	24.654	13.720	24.988	11.320	11.412	11.071	0.001	0.212	0.438	1.000	0.863	0.738	0	0	0	-
5B	14.894	12.948	14.698	25.080	25.605	25.354	14.180	25.346	10.187	12.657	10.656	-1.133	1.457	0.022	2.193	0.364	0.985	0	1	0	-
5C	14.165	12.737	13.186	25.482	25.113	24.859	13.363	25.151	11.317	12.376	11.672	-0.002	1.176	1.039	1.002	0.442	0.487	0	1	1	OK
5D	13.954	13.067	13.742	25.058	24.668	24.950	13.588	24.892	11.104	11.601	11.208	-0.215	0.401	0.574	1.161	0.757	0.672	0	0	0	-
5E	13.188	14.035	13.728	25.188	25.217	24.629	13.650	25.011	12.000	11.183	10.901	0.680	-0.017	0.267	0.624	1.012	0.831	0	0	0	-
5F	13.132	13.007	14.664	25.238	24.795	24.872	13.601	24.968	12.107	11.788	10.208	0.787	0.588	-0.426	0.579	0.665	1.344	0	0	0	-
5G	13.079	13.042	13.967	25.192	24.890	24.750	13.362	24.944	12.114	11.848	10.783	0.795	0.648	0.150	0.577	0.638	0.902	0	0	0	-
5H	13.318	12.884	13.776	25.089	24.974	24.705	13.326	24.922	11.771	12.090	10.929	0.452	0.890	0.295	0.731	0.540	0.815	0	0	0	-
6A	13.693	12.970	12.847	24.788	24.826	24.436	13.170	24.683	11.096	11.855	11.589	-0.224	0.656	0.955	1.168	0.635	0.516	0	0	0	-
6B	13.570	13.251	13.503	25.300	25.198	25.148	13.441	25.215	11.730	11.947	11.645	0.411	0.747	1.011	0.752	0.596	0.496	0	0	1	-
6C	14.169	13.984	15.023	24.951	24.734	24.675	14.392	24.787	10.783	10.749	9.651	-0.537	-0.451	-0.982	1.451	1.367	1.976	0	0	0	-
6D	14.063	13.282	14.230	25.218	25.065	24.733	13.858	25.005	11.154	11.783	10.503	-0.165	0.583	-0.131	1.121	0.668	1.095	0	0	0	-
6E	13.996	12.978	13.881	25.329	25.471	25.159	13.618	25.320	11.333	12.493	11.278	0.014	1.294	0.644	0.991	0.408	0.640	0	1	0	-
6F	12.914	13.589	13.963	25.530	25.272	24.974	13.489	25.259	12.616	11.682	11.011	1.297	0.483	0.378	0.407	0.716	0.770	0	0	0	-
6G	13.917	13.792	14.110	25.484	24.901	25.052	13.940	25.146	11.566	11.109	10.942	0.247	-0.091	0.308	0.843	1.065	0.808	0	1	0	-
6H	13.487	12.943	13.317	25.295	25.123	24.389	13.249	24.936	11.808	12.180	11.072	0.489	0.980	0.439	0.713	0.507	0.738	0	0	0	-
7A	13.707	13.563	14.262	25.641	24.790	25.445	13.844	25.292	11.935	12.050	11.183	0.615	0.027	0.550	0.653	0.981	0.683	0	0	0	-
7B	13.312	13.054	12.959	25.485	25.104	25.797	13.108	25.462	12.173	12.050	12.838	0.854	0.851	2.204	0.553	0.555	0.217	0	0	1	-
7C	13.551	13.618	13.234	25.189	25.247	25.352	13.468	25.263	11.638	11.629	12.118	0.319	0.429	1.484	0.802	0.743	0.357	0	0	1	-
7D	13.552	13.435	12.889	25.160	25.035	24.835	13.292	25.010	11.609	11.600	11.945	0.289	0.400	1.312	0.818	0.758	0.403	0	0	1	-
7E	14.333	14.985	14.894	25.198	25.289	24.875	14.737	25.121	10.866	10.304	9.981	-0.454	-0.896	-0.653	1.369	1.861	1.572	0	0	0	-
7F	13.816	12.613	14.140	25.834	25.292	25.173	13.523	25.433	12.018	12.679	11.033	0.698	1.479	0.399	0.616	0.359	0.758	0	1	0	-
7G	14.139	13.632	14.952	25.126	25.102	25.507	14.241	25.245	10.987	11.470	10.555	-0.332	0.270	-0.079	1.259	0.829	1.056	0	0	0	-
7H	13.215	13.095	13.226	25.216	24.985	24.748	13.178	24.983	12.002	11.890	11.522	0.682	0.690	0.889	0.623	0.620	0.540	0	0	0	-
8A	14.072	14.047	14.437	25.328	24.840	24.495	14.185	24.888													
8B	13.733	13.531	13.822	24.989	24.323	25.103	13.695	24.805													
8C	14.174	13.978	13.989	25.559	24.995	24.031	14.047	24.862													
8D	14.345	12.857	13.992	25.726	25.055	25.144	13.731	25.308													
8E	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8F	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8G	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8H	Undetermine	Undetermine	29.069	Undetermine	Undetermine	Undetermined															

Average Sample 0			
	I	II	III
	14.081	13.604	14.060

Average			
	I	II	III
	25.400	24.803	24.694

Average		
	U6	182
	13.915	24.966

dCT		
I	II	III
11.319	11.200	10.634

ddCT = [(CT 182/31/30d) -[U6]) sample x - [(CT 182/31/30d) -[U6]) sample O

S5 *In cell* screening detail calculation for hsa-miR-182

Sample	U6			hsa-miR-31			Average		dCT 31-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	31	I	II	III	I	II	III	I	II	III	I	II	III	
2A	13.656	12.798	13.272	19.008	19.092	18.987	13.242	19.029	5.351	6.294	5.715	0.328	0.745	0.942	0.797	0.597	0.521	0	0	0	-
2B	13.685	13.517	13.729	18.972	21.173	19.516	13.644	19.887	5.287	7.656	5.787	0.264	2.107	1.013	0.833	0.232	0.495	0	1	1	OK
2C	13.212	13.819	14.066	18.959	18.952	19.131	13.699	19.014	5.747	5.133	5.065	0.723	-0.416	0.292	0.606	1.334	0.817	0	0	0	-
2D	12.777	12.297	13.451	19.052	19.171	19.071	12.842	19.098	6.275	6.875	5.620	1.251	1.326	0.847	0.420	0.399	0.556	1	1	0	OK
2E	13.040	13.680	13.571	18.966	20.298	18.917	13.431	19.394	5.925	6.618	5.346	0.901	1.069	0.572	0.535	0.477	0.673	0	1	0	-
2F	13.849	12.478	13.550	18.924	19.048	19.034	13.292	19.002	5.076	6.570	5.484	0.052	1.021	0.711	0.965	0.493	0.611	0	1	0	-
2G	14.007	13.920	13.483	18.906	18.757	18.784	13.803	18.816	4.899	4.836	5.302	-0.125	-0.713	0.528	1.091	1.639	0.693	0	0	0	-
2H	13.175	13.311	13.875	18.872	19.230	19.790	13.454	19.297	5.697	5.918	5.915	0.673	0.369	1.141	0.627	0.774	0.453	0	0	1	-
3A	13.124	12.947	12.899	19.042	18.777	19.091	12.990	18.970	5.918	5.829	6.192	0.894	0.280	1.418	0.538	0.823	0.374	0	0	1	-
3B	14.250	14.032	14.224	19.498	19.076	19.275	14.169	19.283	5.247	5.044	5.051	0.224	-0.505	0.278	0.856	1.419	0.825	0	0	0	-
3C	13.469	13.456	13.932	19.042	18.851	18.920	13.619	18.937	5.573	5.394	4.988	0.549	-0.155	0.214	0.683	1.113	0.862	0	0	0	-
3D	13.160	12.202	13.578	19.194	19.051	19.245	12.980	19.164	6.035	6.849	5.667	1.011	1.300	0.894	0.496	0.406	0.538	1	1	0	OK
3E	13.160	13.227	13.737	18.820	18.755	18.848	13.375	18.808	5.660	5.528	5.111	0.636	-0.021	0.337	0.644	1.015	0.792	0	0	0	-
3F	13.881	12.711	14.667	18.916	19.137	19.094	13.753	19.049	5.035	6.427	4.427	0.011	0.878	-0.347	0.993	0.544	1.272	0	0	0	-
3G	13.061	13.285	14.196	18.899	18.880	18.823	13.514	18.867	5.838	5.595	4.627	0.814	0.046	-0.147	0.569	0.969	1.107	0	0	0	-
3H	13.312	13.157	13.875	18.818	18.376	18.700	13.448	18.631	5.506	5.218	4.825	0.483	-0.331	0.052	0.716	1.258	0.965	0	0	0	-
4A	13.546	13.598	13.624	19.093	19.035	18.968	13.589	19.032	5.547	5.437	5.344	0.523	-0.112	0.571	0.696	1.081	0.673	0	0	0	-
4B	14.024	13.883	13.925	19.229	18.768	19.185	13.944	19.061	5.205	4.885	5.260	0.181	-0.664	0.486	0.882	1.585	0.714	0	0	0	-
4C	12.975	12.894	12.776	19.204	18.900	19.119	12.882	19.074	6.229	6.007	6.343	1.205	0.458	1.569	0.434	0.728	0.337	1	0	1	OK
4D	14.252	13.858	13.813	19.178	18.953	19.365	13.975	19.165	4.926	5.094	5.552	-0.098	-0.455	0.778	1.070	1.371	0.583	0	0	0	-
4E	14.070	13.183	13.687	19.123	18.707	18.808	13.647	18.879	5.053	5.524	5.121	0.029	-0.025	0.348	0.980	1.018	0.786	0	0	0	-
4F	13.528	13.163	13.455	18.799	18.912	19.259	13.382	18.990	5.271	5.749	5.804	0.247	0.200	1.030	0.843	0.870	0.490	0	0	1	-
4G	14.286	13.824	14.022	19.269	18.915	19.041	14.044	19.075	4.982	5.092	5.019	-0.041	-0.457	0.245	1.029	1.373	0.844	0	0	0	-
4H	12.958	13.353	12.901	18.977	18.437	18.848	13.071	18.754	6.019	5.083	5.947	0.996	-0.466	1.173	0.502	1.381	0.443	0	0	1	-
5A	13.886	13.692	13.583	19.278	19.354	19.165	13.720	19.266	5.392	5.662	5.582	0.368	0.113	0.808	0.775	0.925	0.571	0	0	0	-
5B	14.894	12.948	14.698	19.127	19.389	19.368	14.180	19.295	4.233	6.441	4.670	-0.790	0.892	-0.104	1.730	0.539	1.074	0	0	0	-
5C	14.165	12.737	13.186	18.874	18.983	18.916	13.363	18.924	4.708	6.245	5.730	-0.315	0.696	0.956	1.244	0.617	0.515	0	0	0	-
5D	13.954	13.067	13.742	19.063	19.108	19.188	13.588	19.120	5.109	6.040	5.446	0.085	0.491	0.672	0.943	0.711	0.627	0	0	0	-
5E	13.188	14.035	13.728	19.030	19.150	18.996	13.650	19.059	5.842	5.115	5.268	0.818	-0.434	0.494	0.567	1.351	0.710	0	0	0	-
5F	13.132	13.007	14.664	18.887	18.838	19.347	13.601	19.024	5.755	5.831	4.682	0.731	0.282	-0.091	0.602	0.823	1.065	0	0	0	-
5G	13.079	13.042	13.967	18.718	18.756	18.949	13.362	18.808	5.640	5.714	4.982	0.616	0.165	0.209	0.653	0.892	0.865	0	0	0	-
5H	13.318	12.884	13.776	18.770	18.758	20.360	13.326	19.296	5.452	5.874	6.584	0.428	0.325	1.810	0.743	0.798	0.285	0	0	1	-
6A	13.693	12.970	12.847	19.068	19.164	19.154	13.170	19.129	5.376	6.193	6.307	0.352	0.644	1.533	0.784	0.640	0.346	0	0	1	-
6B	13.570	13.251	13.503	19.434	19.060	19.049	13.441	19.181	5.864	5.809	5.546	0.840	0.260	0.773	0.559	0.835	0.585	0	0	0	-
6C	14.169	13.984	15.023	19.205	18.926	19.132	14.392	19.088	5.036	4.942	4.109	0.012	-0.607	-0.665	0.992	1.523	1.586	0	0	0	-
6D	14.063	13.282	14.230	19.159	18.973	19.150	13.858	19.094	5.096	5.691	4.920	0.072	0.142	0.147	0.951	0.906	0.903	0	0	0	-
6E	13.996	12.978	13.881	19.247	18.979	18.741	13.618	18.989	5.251	6.001	4.860	0.227	0.452	0.086	0.854	0.731	0.942	0	0	0	-
6F	12.914	13.589	13.963	19.220	18.814	18.956	13.489	18.997	6.306	5.224	4.993	1.282	-0.325	0.220	0.411	1.252	0.859	1	0	0	-
6G	13.917	13.792	14.110	18.934	18.707	18.987	13.940	18.876	5.016	4.915	4.876	-0.008	-0.634	0.103	1.005	1.552	0.931	0	0	0	-
6H	13.487	12.943	13.317	18.926	19.040	18.863	13.249	18.943	5.440	6.096	5.546	0.416	0.547	0.773	0.750	0.684	0.585	0	0	0	-
7A	13.707	13.563	14.262	19.017	19.147	18.977	13.844	19.047	5.310	5.584	4.715	0.286	0.035	-0.058	0.820	0.976	1.041	0	0	0	-
7B	13.312	13.054	12.959	19.713	19.005	18.947	13.108	19.222	6.401	5.951	5.988	1.377	0.402	1.215	0.385	0.757	0.431	1	0	1	OK
7C	13.551	13.618	13.234	18.982	19.111	18.981	13.468	19.025	5.431	5.493	5.746	0.407	-0.056	0.973	0.754	1.040	0.510	0	0	0	-
7D	13.552	13.435	12.889	18.966	19.072	18.973	13.292	19.004	5.414	5.636	6.084	0.391	0.087	1.310	0.763	0.941	0.403	0	0	1	-
7E	14.333	14.985	14.894	18.717	18.911	19.057	14.737	18.895	4.384	3.926	4.163	-0.640	-1.623	-0.611	1.558	3.081	1.527	0	0	0	-
7F	13.816	12.613	14.140	18.819	19.113	19.321	13.523	19.084	5.003	6.499	5.181	-0.021	0.950	0.408	1.014	0.517	0.754	0	0	0	-
7G	14.139	13.632	14.952	18.664	18.985	19.038	14.241	18.896	4.525	5.354	4.086	-0.499	-0.196	-0.688	1.413	1.145	1.611	0	0	0	-
7H	13.215	13.095	13.226	18.904	18.640	22.696	13.178	20.080	5.690	5.544	9.471	0.666	-0.005	4.697	0.630	1.003	0.039	0	0	1	-
8A	14.072	14.047	14.437	19.056	19.320	19.153	14.185	19.177													
8B	13.733	13.531	13.822	19.067	19.081	19.097	13.695	19.082													
8C	14.174	13.978	13.989	19.087	19.185	18.797	14.047	19.023													
8D	14.345	12.857	13.992	19.210	19.024	18.285	13.731	18.840													
8E	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8F	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8G	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8H	Undetermine	Undetermine	29.069	Undetermine	Undetermine	Undetermined															

ddCT = ([CT 182/31/30d] - [U6]) sample x - ([CT 182/31/30d] - [U6]) sample O

	Average								dCT		
Average	I	II	III	I	II	III	U6	31	I	II	III
Sample 0	14.081	13.604	14.060	19.105	19.153	18.833	13.915	19.030	5.024	5.549	4.774

S6 *In cell* screening detail calculation for hsa-miR-31

Sample	U6			hsa-miR-30d			Average	dCT 30d-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	30d	I	II	III	I	II	III	I	II	III	I	II	III	
2A	13.656	12.798	13.272	22.071	21.973	22.537	13.242	22.194	8.415	9.175	9.265	0.316	0.290	0.881	0.804	0.818	0.543	0	0	0	0
2B	13.685	13.517	13.729	22.352	22.120	22.920	13.644	22.464	8.668	8.603	9.190	0.568	-0.281	0.806	0.674	1.215	0.572	0	0	0	0
2C	13.212	13.819	14.066	22.008	22.169	22.438	13.699	22.205	8.795	8.350	9.372	0.696	-0.535	-0.012	0.617	1.449	1.008	0	0	0	0
2D	12.777	12.297	13.451	22.147	22.163	22.695	12.842	22.335	9.369	9.866	9.244	1.270	0.982	0.860	0.415	0.506	0.551	1	0	0	0
2E	13.040	13.680	13.571	21.867	22.006	22.029	13.431	22.967	8.826	8.326	8.457	0.727	-0.559	0.073	0.604	1.473	0.951	0	0	0	0
2F	13.849	12.478	13.550	21.975	22.232	22.351	13.292	22.186	8.383	9.497	8.801	0.284	0.612	-0.865	0.821	0.654	0.749	0	0	0	0
2G	14.007	13.920	13.483	22.599	22.940	22.437	13.803	22.325	8.592	8.020	8.954	0.492	-0.865	0.570	0.711	1.821	0.674	0	0	0	0
2H	13.175	13.311	13.875	22.407	22.137	22.330	13.454	22.291	9.232	8.826	8.455	1.132	-0.059	0.071	0.456	1.042	0.952	1	0	0	0
3A	13.124	12.947	12.899	21.971	21.753	22.233	12.990	21.986	8.847	8.806	9.334	0.748	-0.482	-0.079	0.595	1.056	0.518	0	0	0	0
3B	14.250	14.032	14.224	21.868	22.621	22.739	14.169	22.409	7.617	8.590	8.515	-0.482	-0.295	0.131	1.397	1.227	0.913	0	0	0	0
3C	13.469	13.456	13.932	22.097	22.037	22.639	13.619	22.258	8.628	8.581	8.707	0.529	-0.304	0.322	0.693	1.234	0.800	0	0	0	0
3D	13.160	12.202	13.578	22.139	22.001	24.234	12.980	22.792	8.980	9.799	10.656	0.880	0.915	2.272	0.543	0.531	0.207	0	0	1	0
3E	13.160	13.227	13.737	21.823	21.909	22.433	13.375	22.055	8.663	8.682	8.696	0.563	-0.202	0.312	0.677	1.151	0.805	0	0	0	0
3F	13.881	12.711	14.667	22.069	22.018	22.200	13.753	22.096	8.188	9.308	7.533	0.088	0.423	-0.851	0.941	0.746	1.803	0	0	0	0
3G	13.061	13.285	14.196	21.846	21.864	22.377	13.514	22.029	8.785	8.579	8.181	0.685	-0.305	-0.203	0.622	1.236	1.151	0	0	0	0
3H	13.312	13.157	13.875	22.136	21.973	22.071	13.448	22.060	8.824	8.815	8.196	0.725	-0.069	-0.188	0.805	1.049	1.139	0	0	0	0
4A	13.546	13.598	13.624	21.865	22.097	22.435	13.589	22.132	8.320	8.499	8.811	0.220	-0.386	0.427	0.858	1.307	0.744	0	0	1	0
4B	14.024	13.883	13.925	21.951	22.348	22.687	13.944	22.329	7.927	8.465	8.762	-0.172	-0.420	0.378	1.127	1.338	0.769	0	0	0	0
4C	12.975	12.894	12.776	21.949	21.980	22.497	12.882	22.142	8.973	9.086	9.722	0.874	0.202	1.338	0.546	0.870	0.396	0	0	0	0
4D	14.252	13.858	13.813	23.206	22.129	22.393	13.975	22.576	8.954	8.271	8.580	0.854	-0.614	0.196	0.553	1.530	0.873	0	0	0	0
4E	14.070	13.183	13.687	22.148	22.085	22.453	13.647	22.229	8.078	8.902	8.766	-0.022	0.017	0.382	1.015	0.988	0.767	0	0	0	0
4F	13.528	13.163	13.455	21.694	21.737	22.388	13.382	21.940	8.166	8.574	8.933	0.067	-0.310	0.549	0.955	1.240	0.684	0	0	0	0
4G	14.286	13.824	14.022	22.131	22.090	22.409	14.044	22.210	7.845	8.267	8.387	-0.254	-0.618	0.003	1.193	1.535	0.998	0	0	0	0
4H	12.958	13.353	12.901	22.099	22.075	21.809	13.071	21.994	9.142	8.722	8.907	1.042	-0.163	0.523	0.486	1.119	0.696	1	0	0	0
5A	13.886	13.692	13.583	22.168	22.048	22.468	13.720	22.228	8.282	8.356	8.885	0.182	-0.528	0.501	0.881	1.442	0.707	0	0	0	0
5B	14.894	14.894	14.698	22.128	22.252	22.724	14.180	22.368	7.235	9.304	8.026	-0.865	0.419	-0.358	1.821	0.748	1.281	0	0	0	0
5C	14.165	12.737	13.186	21.936	22.418	22.307	13.363	22.220	7.770	9.681	9.121	-0.329	0.796	0.737	1.256	0.576	0.600	0	0	0	0
5D	13.954	13.067	13.742	23.313	21.937	22.639	13.588	22.630	9.360	8.870	8.897	1.260	-0.015	0.513	0.417	1.010	0.701	1	0	0	0
5E	13.188	14.035	13.728	22.111	22.413	22.436	13.650	22.320	8.923	8.379	8.707	0.824	-0.506	0.323	0.565	1.420	0.799	0	0	0	0
5F	13.132	13.007	14.664	22.009	21.968	22.309	13.601	22.095	8.878	8.961	7.645	0.778	0.076	-0.739	0.583	0.949	1.670	0	0	0	0
5G	13.079	13.042	13.967	22.044	22.096	22.211	13.362	22.117	8.965	9.054	8.244	0.866	0.169	-0.140	0.549	0.890	1.102	0	0	0	0
5H	13.318	12.884	13.776	21.908	22.238	21.992	13.326	22.046	8.590	9.354	8.215	0.491	0.470	-0.169	0.712	0.722	1.124	0	0	0	0
6A	13.693	12.970	12.847	21.877	22.299	22.375	13.170	22.184	8.185	9.329	9.528	0.086	0.444	1.143	0.942	0.735	0.453	0	0	0	0
6B	13.570	13.251	13.503	21.934	22.279	22.363	13.441	22.192	8.364	9.028	8.860	0.265	0.143	0.476	0.832	0.906	0.719	0	0	0	0
6C	14.169	13.984	15.023	22.016	22.537	22.461	14.392	22.338	7.847	8.552	7.438	-0.252	-0.333	-0.946	1.191	1.259	1.927	0	0	0	0
6D	14.063	13.282	14.230	22.067	22.375	22.542	13.858	22.328	8.004	9.093	8.312	-0.096	0.208	-0.072	1.068	0.865	1.051	0	0	0	0
6E	13.996	12.978	13.881	22.247	22.524	22.469	13.618	22.414	8.251	9.546	8.588	0.152	0.662	0.204	0.900	0.632	0.868	0	0	0	0
6F	12.914	13.589	13.963	22.128	22.159	22.270	13.489	22.186	9.214	8.570	8.307	1.114	-0.315	-0.077	0.462	1.244	1.055	1	0	0	0
6G	13.917	13.792	14.110	22.186	22.201	22.079	13.940	22.048	7.945	8.409	7.969	-0.154	-0.476	-0.415	1.113	1.391	1.334	0	0	0	0
6H	13.487	12.943	13.317	22.186	30.791	22.159	13.249	25.045	8.699	17.848	8.842	0.600	8.963	0.458	0.660	0.002	0.728	0	1	0	0
7A	13.707	13.563	14.262	22.966	22.156	22.712	13.844	22.611	9.260	8.593	8.450	1.160	-0.292	0.066	0.447	1.224	0.955	1	0	0	0
7B	13.312	13.054	12.959	22.234	22.359	22.498	13.108	22.363	8.921	9.304	9.539	0.822	0.420	1.155	0.566	0.748	0.449	0	0	0	0
7C	13.551	13.618	13.234	21.906	22.025	22.701	13.468	22.210	8.355	8.407	9.466	0.256	-0.478	1.082	0.838	1.393	0.472	0	0	1	0
7D	13.552	13.435	12.889	22.111	22.188	22.404	14.737	22.234	8.559	8.753	9.515	0.460	-0.132	1.130	0.727	1.096	0.457	0	0	1	0
7E	14.333	14.985	14.894	22.115	22.269	22.422	14.737	22.269	7.783	7.284	7.527	-0.317	-1.601	-0.857	1.245	3.033	1.811	0	0	0	0
7F	13.816	12.613	14.140	21.936	21.856	22.194	13.523	21.995	8.120	9.243	8.054	0.021	0.358	-0.330	0.986	0.780	1.257	0	0	0	0
7G	14.139	13.632	14.952	21.892	22.310	22.178	14.241	22.127	7.753	8.678	7.226	-0.346	-0.206	-1.158	1.271	1.154	2.232	0	0	0	0
7H	13.215	13.095	13.226	22.043	21.953	22.017	13.178	22.005	8.829	8.858	8.792	0.729	-0.027	0.407	0.603	1.019	0.754	0	0	0	0
8A	14.072	14.047	14.437	22.066	22.203	22.024	14.185	22.098													
8B	13.733	13.531	13.822	21.922	22.459	22.662	13.695	22.348													
8C	14.174	13.978	13.989	22.555	22.976	22.744	14.047	22.758													
8D	14.345	12.857	13.992	22.179	22.315	22.345	13.731	22.280													
8E	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8F	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8G	Undetermine	Undetermine	Undetermine	Undetermine	Undetermine	Undetermined															
8H	Undetermine	Undetermine	29.069	Undetermine	Undetermine	Undetermined															

Average Sample 0	dCT		
	I	II	III
	14.081	13.604	14.060

Average					
U6		182	dCT		
13.915		22.371	I	II	III
			8.099	8.885	8.384

ddCT = [(CT 182/31/30d] - [U6]) sample x - ((CT 182/31/30d] - [U6]) sample 0

S7 *In cell* screening detail calculation for hsa-miR-30d

Sample	U6			hsa-miR-182			Average		dCT 182-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	182	I	II	III	I	II	III	I	II	III	I	II	III	
3F.	12.693	13.033	13.389	24.219	24.560	23.935	13.038	24.238	11.526	11.527	10.546	1.435	0.083	0.597	0.370	0.944	0.661	1	0	0	-
3G.	13.053	13.221	13.758	24.102	25.166	24.499	13.344	24.589	11.049	11.945	10.741	0.958	0.500	0.792	0.515	0.707	0.577	0	0	0	-
4A.	12.722	13.177	13.606	24.130	24.567	24.169	13.169	24.289	11.408	11.390	10.563	1.317	-0.055	0.614	0.401	1.039	0.653	1	0	0	-
4C.	12.793	13.574	13.579	24.621	23.769	24.227	13.315	24.206	11.828	10.195	10.648	1.738	-1.249	0.699	0.300	2.377	0.616	1	0	0	-
4E.	12.767	14.211	13.885	24.339	23.905	24.425	13.621	24.223	11.572	9.694	10.539	1.482	-1.750	0.590	0.358	3.365	0.664	1	0	0	-
4F.	13.659	14.144	13.480	24.379	23.670	24.009	13.761	24.019	10.720	9.526	10.528	0.629	-1.918	0.579	0.646	3.780	0.669	0	0	0	-
4G.	13.752	14.099	14.411	24.546	23.841	24.248	14.087	24.212	10.795	9.742	9.837	0.704	-1.703	-0.111	0.614	3.255	1.080	0	0	0	-
5A.	14.625	16.062	13.626	24.797	24.144	24.167	14.771	24.370	10.173	8.082	10.541	0.082	-3.362	0.592	0.945	10.284	0.663	0	0	0	-
5G.	13.837	13.240	13.855	23.990	24.652	23.894	13.644	24.179	10.153	11.412	10.039	0.063	-0.032	0.090	0.957	1.023	0.940	0	0	0	-
6A.	14.315	13.934	13.800	24.659	24.845	24.601	14.016	24.702	10.344	10.911	10.802	0.253	-0.533	0.853	0.839	1.447	0.554	0	0	0	-
6B.	13.911	13.833	13.480	24.304	24.299	23.891	13.741	24.165	10.394	10.466	10.411	0.303	-0.978	0.462	0.811	1.970	0.726	0	0	0	-
6C.	13.261	14.380	14.723	24.968	23.897	23.982	14.121	24.282	11.707	9.517	9.259	1.617	-1.927	-0.690	0.326	3.803	1.613	1	0	0	-
6E.	13.817	14.653	14.068	24.270	24.771	24.172	14.179	24.404	10.453	10.118	10.104	0.363	-1.327	0.156	0.778	2.508	0.898	0	0	0	-
6F.	14.038	15.354	13.932	24.307	24.012	23.815	14.441	24.045	10.269	8.658	9.884	0.178	-2.786	-0.065	0.884	6.897	1.046	0	0	0	-
8C.	13.941	15.029	14.231	24.123	24.484	24.412	14.400	24.340	10.182	9.455	10.181	0.091	-1.989	0.232	0.939	3.971	0.851	0	0	0	-
9A.	13.472	14.950	14.227	24.601	24.438	24.504	14.216	24.514	11.129	9.488	10.277	1.039	-1.957	0.328	0.487	3.881	0.797	1	0	0	-
10A.	13.211	12.768	13.786	24.079	24.716	24.126	13.255	24.307	10.868	11.948	10.341	0.777	0.504	0.392	0.583	0.705	0.762	0	0	0	-
10B.	14.454	13.806	13.763	24.427	25.146	23.923	14.008	24.499	9.974	11.340	10.160	-0.117	-0.105	0.211	1.084	1.075	0.864	0	0	0	-
10D.	14.432	13.954	13.872	24.214	24.850	23.879	14.086	24.314	9.781	10.896	10.006	-0.309	-0.548	0.058	1.239	1.462	0.961	0	0	0	-
Sample 0	15.058	13.158	14.322	25.148	24.602	24.271	14.179	24.674	10.090	11.444	9.949	0.000	0.000	0.000	1.000	1.000	1.000	0	0	0	

S8 Additional *In cell* Assay detail calculation for hsa-miR-182

Sample	U6			hsa-miR-31			Average		dCT 31-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	31	I	II	III	I	II	III	I	II	III	I	II	III	
3F.	12.693	13.033	13.389	18.193	17.998	17.979	13.038	18.057	5.500	4.965	4.590	2.041	-0.073	0.450	0.2430	1.0520	0.7322	1	0	0	-
3G.	13.053	13.221	13.758	18.098	18.192	18.068	13.344	18.119	5.045	4.971	4.310	1.586	-0.067	0.169	0.3331	1.0474	0.8892	1	0	0	-
4A.	12.722	13.177	13.606	18.049	18.200	18.280	13.169	18.176	5.327	5.022	4.674	1.869	-0.016	0.533	0.2738	1.0111	0.6910	1	0	0	-
4C.	12.793	13.574	13.579	18.140	18.135	18.161	13.315	18.145	5.348	4.562	4.581	1.889	-0.476	0.441	0.2700	1.3910	0.7369	1	0	0	-
4E.	12.767	14.211	13.885	18.175	18.258	18.548	13.621	18.327	5.408	4.047	4.663	1.949	-0.991	0.522	0.2589	1.9877	0.6965	1	0	0	-
4F.	13.659	14.144	13.480	18.268	18.235	18.492	13.761	18.332	4.610	4.091	5.011	1.151	-0.947	0.871	0.4503	1.9277	0.5469	1	0	0	-
4G.	13.752	14.099	14.411	18.440	18.132	18.456	14.087	18.342	4.688	4.033	4.045	1.229	-1.005	-0.096	0.4265	2.0075	1.0690	1	0	0	-
5A.	14.625	16.062	13.626	18.809	18.300	18.465	14.771	18.525	4.184	2.238	4.839	0.726	-2.800	0.698	0.6048	6.9646	0.6163	0	0	0	-
5G.	13.837	13.240	13.855	18.323	18.285	18.085	13.644	18.231	4.486	5.045	4.229	1.028	0.007	0.089	0.4905	0.9952	0.9404	1	0	0	-
6A.	14.315	13.934	13.800	18.602	18.284	18.393	14.016	18.427	4.287	4.350	4.593	0.829	-0.688	0.453	0.5631	1.6110	0.7307	0	0	0	-
6B.	13.911	13.833	13.480	18.198	18.173	18.132	13.741	18.168	4.288	4.340	4.652	0.829	-0.697	0.511	0.5629	1.6217	0.7017	0	0	0	-
6C.	13.261	14.380	14.723	18.123	18.134	18.215	14.121	18.157	4.862	3.754	3.492	1.404	-1.284	-0.649	0.3779	2.4349	1.5680	1	0	0	-
6E.	13.817	14.653	14.068	18.392	18.037	18.636	14.179	18.355	4.575	3.383	4.569	1.117	-1.655	0.428	0.4612	3.1485	0.7434	1	0	0	-
6F.	14.038	15.354	13.932	18.663	18.190	18.370	14.441	18.408	4.625	2.837	4.438	1.167	-2.201	0.298	0.4454	4.5984	0.8136	1	0	0	-
8C.	13.941	15.029	14.231	18.559	18.232	18.549	14.400	18.446	4.618	3.203	4.318	1.159	-1.835	0.177	0.4478	3.5681	0.8847	1	0	0	-
9A.	13.472	14.950	14.227	18.953	18.465	18.717	14.216	18.712	5.482	3.515	4.490	2.023	-1.523	0.349	0.2461	2.8731	0.7849	1	0	0	-
10A.	13.211	12.768	13.786	18.190	17.949	18.147	13.255	18.095	4.979	5.181	4.361	1.520	0.143	0.220	0.3487	0.9054	0.8583	1	0	0	-
10B.	14.454	13.806	13.763	18.141	18.094	18.424	14.008	18.220	3.688	4.288	4.660	0.229	-0.750	0.519	0.8532	1.6817	0.6976	0	0	0	-
10D.	14.432	13.954	13.872	18.616	17.943	18.461	14.086	18.340	4.184	3.989	4.588	0.725	-1.049	0.447	0.6048	2.0690	0.7333	0	0	0	-
Sample 0	15.058	13.158	14.322	18.516	18.196	18.463	14.179	18.391	3.459	5.038	4.141	0.000	0.000	0.000	1.0000	1.0000	1.0000	0	0	0	

S9 Additional *In cell* Assay detail calculation for hsa-miR-31

Sample	U6			hsa-miR-30d			Average		dCT 30d-U6			ddCT (dCT sample -dCT 0)			2^-ddCT			Score X<0.5			R
	I	II	III	I	II	III	U6	30d	I	II	III	I	II	III	I	II	III	I	II	III	
3F.	12.693	13.033	13.389	23.001	23.487	23.481	13.038	23.323	10.308	10.454	10.093	1.397	0.217	0.625	0.3797	0.8603	0.6484	1	0	0	-
3G.	13.053	13.221	13.758	22.979	23.408	23.491	13.344	23.293	9.926	10.187	9.733	1.014	-0.050	0.265	0.4951	1.0354	0.8319	1	0	0	-
4A.	12.722	13.177	13.606	22.853	23.223	23.565	13.169	23.214	10.131	10.046	9.959	1.220	-0.191	0.491	0.4294	1.1419	0.7113	1	0	0	-
4C.	12.793	13.574	13.579	22.988	23.369	23.689	13.315	23.349	10.195	9.796	10.110	1.284	-0.441	0.642	0.4107	1.3578	0.6407	1	0	0	-
4E.	12.767	14.211	13.885	23.300	23.380	23.664	13.621	23.448	10.532	9.169	9.778	1.621	-1.068	0.311	0.3252	2.0967	0.8062	1	0	0	-
4F.	13.659	14.144	13.480	23.262	23.628	23.854	13.761	23.581	9.603	9.484	10.373	0.692	-0.753	0.906	0.6190	1.6857	0.5338	0	0	0	-
4G.	13.752	14.099	14.411	23.404	23.588	23.594	14.087	23.529	9.652	9.489	9.183	0.741	-0.748	-0.285	0.5985	1.6796	1.2183	0	0	0	-
5A.	14.625	16.062	13.626	24.051	32.956	23.641	14.771	26.883	9.426	16.894	10.015	0.515	6.657	0.548	0.7000	0.0099	0.6842	0	1	0	-
5G.	13.837	13.240	13.855	23.376	23.567	23.682	13.644	23.542	9.540	10.327	9.827	0.628	0.090	0.359	0.6470	0.9396	0.7798	0	0	0	-
6A.	14.315	13.934	13.800	23.349	23.026	23.262	14.016	23.212	9.034	9.092	9.462	0.122	-1.145	-0.006	0.9187	2.2119	1.0041	0	0	0	-
6B.	13.911	13.833	13.480	23.533	23.438	23.664	13.741	23.545	9.622	9.606	10.184	0.711	-0.631	0.716	0.6109	1.5489	0.6086	0	0	0	-
6C.	13.261	14.380	14.723	23.342	23.468	23.644	14.121	23.485	10.081	9.088	8.921	1.170	-1.149	-0.546	0.4445	2.2171	1.4604	1	0	0	-
6E.	13.817	14.653	14.068	23.444	23.570	24.002	14.179	23.672	9.627	8.916	9.935	0.715	-1.321	0.467	0.6090	2.4979	0.7235	0	0	0	-
6F.	14.038	15.354	13.932	24.040	23.750	23.710	14.441	23.833	10.002	8.396	9.779	1.091	-1.841	0.311	0.4696	3.5829	0.8060	1	0	0	-
8C.	13.941	15.029	14.231	23.897	23.723	23.824	14.400	23.815	9.956	8.694	9.593	1.045	-1.543	0.125	0.4847	2.9148	0.9168	1	0	0	-
9A.	13.472	14.950	14.227	24.045	24.350	23.991	14.216	24.129	10.573	9.400	9.764	1.662	-0.837	0.296	0.3161	1.7865	0.8143	1	0	0	-
10A.	13.211	12.768	13.786	23.407	23.319	23.545	13.255	23.424	10.196	10.551	9.760	1.284	0.314	0.292	0.4105	0.8044	0.8168	1	0	0	-
10B.	14.454	13.806	13.763	23.912	23.480	23.923	14.008	23.772	9.459	9.673	10.160	0.547	-0.564	0.692	0.6845	1.4782	0.6190	0	0	0	-
10D.	14.432	13.954	13.872	23.925	23.419	23.733	14.086	23.692	9.493	9.465	9.860	0.581	-0.772	0.393	0.6684	1.7080	0.7617	0	0	0	-
Null RT	15.058	13.158	14.322	23.969	23.395	23.789	14.179	23.718	8.912	10.237	9.468	0.000	0.000	0.000	1.0000	1.0000	1.0000	0	0	0	

S10 Additional *In cell* Assay detail calculation for hsa-miR-30d

Substrate 25 nM

Time Sampling (min)	CT mean Value		Conc (uM) mean		**Δ** CT to 0 min
	Ct Value	SD	Conc (uM)	SD	
0	33.488	0.7355	0.00710	7.59.E-06	0.0000
2.5	32.258	0.1585	0.00684	3.53.E-06	1.2301
5	32.156	0.4772	0.00959	9.18.E-06	1.3323
10	30.820	0.0661	0.01216	4.20.E-06	2.6681
20	30.128	0.0519	0.02293	2.63.E-05	3.3602
40	30.147	0.0193	0.02960	1.52.E-05	3.3411
80	29.109	0.0460	0.10214	1.28.E-04	4.3797
180	27.467	0.0142	0.15325	1.61.E-04	6.0209

Substrate 50 nM

Time Sampling (min)	CT mean Value		Conc (uM) mean		**Δ** CT to 0 min
	Ct Value	SD	Conc (uM)	SD	
0	32.855	0.0989	0.00546	3.76.E-05	0
2.5	31.698	0.1315	0.00323	3.15.E-06	1.156601
5	32.045	0.1335	0.00294	1.08.E-05	0.809743
10	31.241	0.1030	0.00473	2.30.E-05	1.613736
20	28.993	0.1065	0.02149	3.23.E-05	3.861771
40	28.943	0.0782	0.02607	2.38.E-05	3.912268
80	27.624	0.0773	0.10497	5.74.E-04	5.230887
180	26.126	0.0885	0.38511	2.24.E-03	6.728572

S11 Detail calculation of 25 nM and 50 nM substrate for velocity plot

Substrate 100 nM

Time Sampling (min)	CT mean Value		Conc (uM) mean		**Δ** CT to 0 min
	Ct Value	SD	Conc (uM)	SD	
0	29.508	0.0486	0.06489	1.43.E-04	0
2.5	30.811	0.0916	0.15440	9.02.E-05	0.32676
5	32.382	0.1918	0.02403	3.15.E-05	-1.19671
10	30.717	0.0719	0.07559	8.01.E-05	-0.30246
20	30.246	0.1046	0.52890	1.47.E-04	1.909919
40	30.136	0.0210	0.89781	2.55.E-04	2.727061
80	29.867	0.1016	0.76997	2.91.E-03	3.364975
180	27.966	0.0899	4.18384	4.63.E-03	5.561035

Substrate 200 nM

Time Sampling (min)	CT mean Value		Conc (uM) mean		**Δ** CT to 0 min
	Ct Value	SD	Conc (uM)	SD	
0	28.963	0.1185	0.00630	1.98.E-02	0
2.5	27.124	0.1121	0.02376	2.06.E-01	1.83901
5	24.958	0.0143	0.07054	1.26.E-01	4.004938
10	24.538	0.0639	0.09258	2.83.E-01	4.425247
20	23.639	0.0941	0.16015	4.96.E-01	5.324462
40	22.217	0.0538	0.37632	9.31.E-01	6.74645
80	22.257	0.0316	0.39424	2.23.E+00	6.705735
180	20.325	0.1173	1.17342	1.07.E+00	8.638033

S12 Detail calculation of 100 nM and 200 nM substrate for velocity plot

Substrate 300 nM

Time Sampling	CT mean Value		Conc (uM) mean		**Δ** CT to 0
(min)	Ct Value	SD	Conc (uM)	SD	min
0	26.884	0.0249	0.25513	4.85.E-04	0
2.5	26.780	0.1028	0.26920	4.11.E-04	0.103791
5	24.641	0.0516	1.03634	1.20.E-03	2.24324
10	26.052	0.0258	0.60742	3.12.E-03	0.831655
20	25.140	0.0174	0.78602	1.79.E-03	1.744209
40	24.442	0.0210	1.21573	2.58.E-03	2.442131
80	24.021	0.0653	1.56371	2.72.E-03	2.862636
180	20.999	0.0176	11.62720	3.76.E-02	5.884878

Substrate 400 nM

Time Sampling	CT mean Value		Conc (uM) mean		**Δ** CT to 0
(min)	Ct Value	SD	Conc (uM)	SD	min
0	29.001	0.0494	0.07517	2.35.E-04	0
2.5	27.045	0.0270	0.37456	2.12.E-03	1.955842
5	23.948	0.0034	1.64587	4.58.E-03	5.052423
10	23.198	0.0114	2.53681	5.79.E-03	5.802208
20	22.416	0.0187	3.92261	3.66.E-03	6.585011
40	24.739	0.0251	0.93875	8.95.E-04	4.262106
80	21.759	0.0095	8.59525	4.47.E-02	7.241521
180	19.526	0.0501	23.16750	1.72.E-02	9.474163

S13 Detail calculation of 300 nM and 400 nM substrate for velocity plot

List of presentations

1. **Muhammad Nurrohman Sidiq**, Asako Murata, and Kazuhiko Nakatani, “Screening of small molecules that interfere dicer-mediated processing of pre-miR-182/31/30d”, Japan Society of Chemical Biology, virtual meeting, June 2021 (Poster)
2. **Muhammad Nurrohman Sidiq**, Asako Murata, and Kazuhiko Nakatani, “qPCR-based screening methods for small molecules that modulate dicer-mediated pre-miR-182/31/30d”, The 48th International Symposium of Nucleic Acids Chemistry, virtual meeting, November 2021 (Poster)