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Corresponding Author:	Jong Kook Park, D.V.M., Ph.D. Hallym University Chuncheon, Gangwon-do KOREA, REPUBLIC OF
Corresponding Author's Institution:	Hallym University
Corresponding Author E-Mail:	jkp555@hallym.ac.kr
Order of Authors:	Hyun Ah Seo
	Cho Yean Hwang
	Sokviseth Moeng
	Jong Kook Park, D.V.M., Ph.D.
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TITLE

An In Vitro Protocol for Evaluating MicroRNA Levels, Functions, and Associated Target Genes in Tumor Cells.

AUTHORS AND AFFILIATIONS

Hyun Ah Seo[*1], Cho Yean Hwang[*1], Sokviseth Moeng[1], Jong Kook Park[1]

[1]Department of Biomedical Science and Research Institute for Bioscience & Biotechnology, Hallym University, Chuncheon, Republic of Korea

[*] These authors contributed equally

Corresponding Author:

Sokviseth Moeng: sokvisethmoeng@yahoo.com

Jong Kook Park: jkp555@hallym.ac.kr

KEYWORDS

microRNA, miRNA-107, real-time PCR, SRB assay, dose-response curve, cloning, luciferase assay

SUMMARY

This protocol uses a probe-based real-time polymerase chain reaction (PCR), a sulforhodamine B (SRB) assay, 3' untranslated regions (3' UTR) cloning, and a luciferase assay to verify the target genes of a miRNA of interest and to understand the functions of miRNAs.

ABSTRACT

MicroRNAs (miRNAs) are small regulatory RNAs which are recognized to modulate numerous intracellular signaling pathways in several diseases including cancers. These small regulatory RNAs mainly interact with the 3' untranslated regions (3' UTR) of their target messenger RNAs (mRNAs) ultimately resulting in the inhibition of decoding processes of mRNAs and the augmentation of target mRNA degradations. Based on the expression levels and intracellular functions, miRNAs are able to serve as regulatory factors of oncogenic and tumor-suppressive mRNAs. Identification of bona fide target genes of a miRNA among hundreds or even thousands of computationally predicted targets is a crucial step to discern the roles and basic molecular mechanisms of a miRNA of interest. Various miRNA target prediction programs are available to search possible miRNA-mRNA interactions. However, the most challenging question is how to validate direct target genes of a miRNA of interest. This protocol describes a reproducible strategy of key methods on how to identify miRNA targets related to the function of a miRNA. This protocol presents a practical guide on step-by-step procedures to uncover miRNA levels, functions, and related target mRNAs using the probe-based real-time polymerase chain reaction (PCR), sulforhodamine B (SRB) assay following a miRNA mimic transfection, dose-response curve generation, and luciferase assay along with the cloning of 3' UTR of a gene, which is necessary for proper understanding of the roles of individual miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are the small regulatory RNAs that mainly modulate the process of translation and degradation of messenger RNAs (mRNAs) by reacting to the 3' untranslated regions (3' UTR) in bona fide target genes[1]. Expression of miRNAs can be regulated by transcriptional and post-transcriptional mechanisms. The imbalance of such regulatory mechanisms brings uncontrolled and distinctive miRNAs expression levels in numerous diseases including cancers[2]. A single miRNA can have multiple interactions with diverse mRNAs. Correspondingly, an individual mRNA can be controlled by various miRNAs. Therefore, intracellular signaling networks are intricately influenced by distinctively expressed miRNAs by which physiological disorders and diseases can be initiated and deteriorated[2-6]. Although the altered expression of miRNAs has been observed in various types of cancers, the molecular mechanisms that modulate the manners of cancer cells in conjunction with miRNAs are still largely unknown.

Accumulating evidence has been showing that the oncogenic or tumor-suppressive roles of miRNAs depend on the types of cancers. For example, by targeting forkhead box o3 (FOXO3), miR-155 promotes the cell proliferation, metastasis, and chemoresistance of colorectal cancer[7,8]. In contrast, the restriction of glioma cell invasion is induced by miR-107 via the regulation of neurogenic locus notch homolog protein 2 (NOTCH2) expression[9]. The assessment of miRNA-target interactions in connection with miRNA functions is an indispensable part to better understand how miRNAs regulate various biological processes in both healthy and diseased states[10]. In addition, the discovery of bona fide target(s) of miRNAs can further provide a fine-tuned strategy for a miRNA-based therapy with various anti-cancer drugs. However, the main challenge in the field of miRNAs is the identification of direct targets of miRNAs. Here, detailed methods are presented as reproducible experimental approaches for the miRNA target gene determination. Successful experimental design for the miRNA target identification involves various steps and considerations (**Figure 1**). Comparison of mature miRNA levels in tumor cells and normal cells can be one of the common procedures to select a miRNA of interest (**Figure 1A**). The functional study of a selected miRNA to detect the effects of a miRNA on cell proliferation is important to narrow down the list of best potential candidate targets of a miRNA of interest (**Figure 1B**). Based on the experimentally validated functions of miRNAs, a systematic review of literature and database in company with a miRNA target prediction program is required to search the most relevant information on gene functions (**Figure 1C**). The identification of real target genes of a miRNA of interest can be achieved by implementing experiments such as the luciferase assay along with the cloning of 3' UTR of a gene, real-time PCR, and western blotting (**Figure 1D**). The goal of the current protocol is to provide comprehensive methods of key experiments, the probe-based real-time polymerase chain reaction (PCR), sulforhodamine B (SRB) assay following a miRNA mimic transfection, dose-response curve generation, and luciferase assay along with the cloning of 3' UTR of a gene. The current protocol can be useful for a better understanding of the functions of individual miRNAs and the implication of a miRNA in cancer therapy.

PROTOCOL

1. Mature microRNA (miRNA) expression analysis

1.1. Mature miRNA complementary DNA (cDNA) synthesis

1.1.1. Add 254 ng of total RNA and 4.5 µL of deoxyribonuclease I (DNase I) mixtures, and then add ultrapure water into PCR strip-tubes to make up to 18 µL (**Figure 2A**). Prepare the reaction for each total RNA sample purified from several cell lines using enough amount of DNase I mixtures based on the total number of reactions.

NOTE: DNase I mixtures are composed of DNase I (1.8 µL), ribonuclease inhibitor (0.3 µL), and 25 mM $MgCl_2$ (2.4 µL). To reproducibly procure total RNA, a column-based extraction method was applicated instead of using a phenol-chloroform based extraction method. It was reported that the extraction yield of some miRNAs can be varied depending on the number of cells when using a phenol-chloroform based extraction method[11,12].

1.1.2. Incubate the tubes in a thermal cycler. Run the tubes for 10 min at 37 °C, and heat-inactivate DNase I by 5 min incubation at 90 °C. Immediately place the tubes on ice after incubation.

1.1.3. Transfer 7.1 µL of DNase I treated total RNA into 2 sets of new tubes and then add 1.5 µL of antisense primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (**Figure 2B**).

NOTE: The amount of total RNA for cDNA synthesis becomes 100 ng at this step. The stock concentration of GAPDH antisense primers is 10 µM. Adding GAPDH antisense primers is for the generation of GAPDH cDNAs using a gene-specific primer method.

1.1.4. Incubate the tubes using a thermal cycler. Start at 80 °C for 5 min followed by the reaction at 60 °C for 5 min. Immediately place the tubes on ice after incubation.

1.1.5. Add 3.4 µL of reverse transcription (RT) enzyme mixtures in each reaction (**Figure 2B**). RT enzyme mixtures are composed of 100 mM deoxyribonucleotide triphosphates (0.15 µL), 10x RT buffer (1.5 µL), ribonuclease inhibitor (0.75 µL), and reverse transcription enzyme (1 µL). Prepare enough amount of mixtures based on the total number of reactions.

1.1.6. Add 3 µL of 5x RT primers for a specific miRNA in each reaction (**Figure 2B**).

NOTE: Total volume is 15 µL for each reaction.

1.1.7. Run the tubes using a thermal cycler. Start at 16 °C for 30 min followed by the reaction at 42 °C for 30 min, and finally at 85 °C for 5 min. Hold at 4 °C for any remaining time (**Figure 2B**). Single-stranded cDNAs are generated in this step for both a specific miRNA and GAPDH gene in the same tube.

1.2. Real-time polymerase chain reaction (PCR) and data analysis

1.2.1. Dilute each cDNA with ultrapure water at 1:49 ratio.

1.2.2. Prepare the reaction mixtures for a specific miRNA and GAPDH (**Table 1**). For the detection of a specific miRNA and GAPDH, set up triplicate reactions for each cDNA sample.

1.2.3. Perform the real-time PCR and data analysis (**Figure 2C**). Analyze data using the comparative C_T method[13,14].

2. MicroRNA (miRNA) mimic transfection

NOTE: miRNA-107 is selected from step 1. Since miRNA-107 is down-regulated in tumor cells compared with normal cells, it can be speculated that miRNA-107 is a tumor suppressive miRNA. In the case of a miRNA which is up-regulated in tumor cells compared with normal cells (e.g., miRNA-301), antisense oligonucleotides against miRNA-301 can be applied for steps 2, 3, and 4.

2.1. Count the cells with a counting-chamber device and plate the cells in a 96-well plate. Cell density is 2 x 10^3 cells/100 µL for each well. Do not use cell culture media containing penicillin-streptomycin (P/S) because P/S can reduce the transfection efficiency.

2.2. Prepare a set of transfection mixtures to transfect the cells at several final concentrations of miRNA control mimic and miRNA-107 mimic on the next day (**Figure 3**).

2.2.1. From the stock (25 µM concentration) of miRNA control mimic or miRNA-107 mimic, dilute and add corresponding amount of control mimic or miRNA-107 mimic in the reduced-serum media along with a transfection reagent using microcentrifuge tubes (**Figure 3A**). Gently mix the oligo containing mixtures using a micropipette. The total amount of oligos (miRNA mimic control + miRNA-107 mimic) should be same in each well. Blank wells include 100 µL of cell culture media and reduced-serum media containing a transfection reagent without cells.

2.3. After a 10 min incubation in a cell culture hood, gently mix the oligo containing mixtures again and then add 50 µL of the mixtures into each well. Keep the transfected cells in a cell culture incubator. Replace the transfection reagent containing media with the fresh cell culture media containing both fetal bovine serum (FBS) and P/S after 6-12 h incubation. Further incubate the cells for 72 h. The total treatment duration of miRNA mimic is 96 h.

3. Sulforhodamine B (SRB) assay

3.1. Cell fixation

3.1.1. Remove the cell culture media in each well of the plate and promptly fill 100 µL of 10% trichloroacetic acid (TCA) into each well. Carefully aspirate the cell culture media from each well to avoid any cell damage and detachment from the bottom.

NOTE: Prepare 40% TCA by adding 20 g of TCA powder into 50 mL of distilled water. From 40%

TCA, make 10% TCA by diluting 40% TCA with distilled water at a 1:3 dilution ratio.

3.1.2. Keep the plate containing 10% TCA in a refrigerator (4 °C) for 1 h.

3.1.3. Wash the plate several times by submerging into the water tub and dry it. Remove excess water from inside of wells by tapping the plate until there is no water left in wells. Leave the plate on a laboratory bench to dry it before going to the next step.

3.2. Cell staining

3.2.1. Pipette 50 µL of 0.4% SRB solution into each well including blank wells. Gently shake the plate until 0.4% SRB solution consistently covers the bottom of wells.

NOTE: Prepare and use 0.4% SRB solution by adding 0.4 g of SRB powder into 100 mL of 1% acetic acid. Shake the solution carefully to mix it. Wrap the bottle of 0.4% SRB solution in a light protective material such as aluminum foil. Store 0.4% SRB solution in a refrigerator.

3.2.2. After incubation for 40 min to 60 min, wash the plate by rinsing it with 1% acetic acid. Wash the plate until the unbound dye is totally washed away (**Figure 3B**).

3.2.3. Leave the plate on a laboratory bench to dry it before going to the next step.

NOTE: The plate should be entirely dried before going to step 3.3.

3.3. Absorbance measurement

3.3.1. Pipette 100 µL of Tris base solution (10 mM) into the corresponding wells including blank wells. Keep the plate on a shaker for 10 min. Measure the absorbance at 492 nm.

4. Generation of a dose-response curve

4.1. Analyze the SRB assay data in a spreadsheet. Subtract the blank absorbance from the absorbance values of each group and calculate the average (AVE) and standard deviation (STD) of absorbance values of each group.

4.2. Calculate the percentage of average absorbance (AVE%) and that of standard deviation (STD%) of each group using absorbance values of the SRB assay.

NOTE: The AVE% of miRNA control mimic treated group is 100%. Calculate the STD% using the following formula: STD% = (STD of each group / AVE absorbance of control mimic treated group) x 100.

4.3. Import the raw data including treatment concentrations, AVE%, and STD% into the software by vertically aligning those data. Since Log 0 is not defined, set the first concentration

of X axis to a value that is close to 0 (e.g., 0.01).

4.4. Click **Create graph** tab and choose **Simple scatter-error bars**. Select **Worksheet columns** as symbol values and click **Next**. In the data format panel, select **XY pairs** and click **Next**. Select corresponding data columns in the select data panel. Click **Finish** button to create the plot.

NOTE: The X axis represents the concentrations, the Y axis indicates the percentage of average absorbance of each concentration (AVE%), and the error bars point out the percentage of standard deviation of each concentration (STD%).

4.5. Double-click on the X axis to modify the type of scale and the scaling of axis. Change the type of scale from linear to log. Modify the start and end range number to 0.01 and 200, respectively.

4.6. Right-click on any scatter plot, choose **Curve fit**, and go to the sub-category **User- defined**. Select **Dose-response curve**, click **Next** buttons, and then click **Finish** button. The dose-response curve is now generated along with a report tab (**Figure 4A**).

4.6.1. To input Equation 1 in the software for the generation of a dose-response curve, click the **Analysis** tab and select **Regression wizard**. Go to **User-defined** in the equation category and then click the **New** button. Insert Equation 1, variables, initial parameters, and constraints into the corresponding blank boxes (**Figure 4B, C**). Click **Add as** button and set the name of the equation as **Dose-response curve**. The equation name is now generated in the sub-category **User-defined** in the equation category. **f** indicates the percentage of cell viability (% cell viability) in Equation 1.

Equation 1

$$\mathrm{f} = (\mathrm{y0} - \mathrm{R}) \times \left(1 - \frac{x^n}{k^n + x^n}\right) + \mathrm{R}$$

4.7. Go to the report tab and then check the n, k, and R values.

NOTE: y0 indicates 100% cell viability of miRNA control mimic treated group, n indicates the Hill-type coefficient (the slope of a plot), k indicates the concentration of miRNA-107 mimic that produces a 50% of the miRNA-107 mimic's maximum effect (the half maximal inhibitory concentration, IC_{50}), and R indicates the residual unaffected fraction (the resistance fraction)[15]. The equation used to generate a dose-response curve recognizes the range from y0 to R value (if any) as 100% (**Figure 4A**). Therefore, it is necessary to acquire the adjusted k (IC_{50}) value that is calculated based on the range from y0 to a value of zero (**Figure 4A**). Adjusted k (IC_{50}) along with other ICx values (e.g., IC_{10} through IC_{90}) can be obtained using Equation 2, which is derived from Equation 1. The derivation of Equation 2 from Equation 1 is indicated in **Supplementary Figure 1**.

Equation 2

$$x = k \times \sqrt[n]{\frac{\mathrm{y0} - f}{f - R}}$$

4.8. Double-click the left mouse button on the cell in which Equation 2 is applied. Using Equation 2 and parameters from the generated dose-response curve, it is available to calculate the adjusted values of ICx, ranging from IC_{10} to IC_{90} (**Figure 4D**).

4.9. Input the equal sign followed by the formula beginning with a bracket in the cell. When entering the formula, fix the value of n, k, and R as the absolute cell references by adding the dollar sign to the corresponding column and row, so that these fixed values will not be changed when auto-filling the formula down to the rows (**Figure 4D**). Alternatively, adjusted values can be manually calculated using Equation 2.

NOTE: IC_{90} value is not determined because the R value is greater than 10. In addition, if R value is above 20, the value of IC_{80} is also not determined (**Figure 4D**).

5. Verification of the direct target gene of a microRNA of interest

NOTE: After performing the functional experiment such as the SRB assay, miRNA-107 is confirmed as a tumor suppressive miRNA and it is highly feasible that miRNA-107 directly targets oncogenes. Check the list of all predicted target genes using a miRNA target prediction program such as TargetScan (http://www.targetscan.org/vert_71/), and then narrow down to potential candidate targets based on the function of a gene in databases including PubMed and GeneCards.

5.1. Primer design for the cloning of 3' untranslated region (UTR)

5.1.1. Put the name of a gene in GeneCards (https://www.genecards.org/) and click **Symbol** of a gene. Assess to Ensembl genome browser by clicking **Ensembl ID** of a gene and then click **Transcripts ID** in the transcript table. After that, click **Exons** existed in the **Transcript-based displays** list on the left.

5.1.2. Copy the nucleotide sequences of the 3' UTR and paste it into the primer design program. Copy the sequences again from this program and paste it into a word processor. Check the presence of miRNA binding sequences as well as the presence of restriction enzymes sites used for the cloning.

NOTE: If there are no restriction enzyme recognition sites within the 3' UTR, the restriction enzymes selected for the cloning can be used for the next step.

5.1.3. In the primer design program, accept the 3' UTR sequences and start to design the forward and reverse primers with the following condition. Length: 20-30 nucleotides, Tm: 45-58 °C, GC%: 40-60%. The difference between the Tm values of the two primers should be less than 5 °C. The primer sequences used in this study are provided in **Supplementary Figure 2**. Add restriction enzyme recognition sequences as well as 4 random nucleotides to the designed primers.

5.2. Gradient PCR

5.2.1. Prepare 25 µL of PCR reaction mixtures including designed primers per one annealing temperature (**Table 2**). Prepare enough amount of mixtures based on the total number of reactions. Mix the solution by pipetting and add 25 µL of reaction mixtures into each tube. Centrifuge the tubes for few seconds.

5.2.2. Perform 35-40 PCR cycles from denaturation step to extension step. Set up the PCR cycle as the following steps: 98 °C for 1 min (1 cycle, polymerase activation step), 95 °C for 10 s (denaturation step), 45 °C-68 °C for 30 s (annealing step), 68 °C (extension step, 10 s-1 min per 1000 bp), 68 °C for 3 min (termination step), and finally cool down to 4 °C.

5.2.3. Run the PCR products and check bands on a 1% agarose gel with DNA ladders. Find the best annealing temperature (**Figure 5A**). Amplify 3' UTR of a gene again using the best annealing temperature for the next step.

5.3. Double digestion

5.3.1. Make the reaction mixtures including two restriction enzymes, XhoI (or AsiSI) and NotI, in a tube (**Table 3**). Incubate the mixtures for 3-4 h using a water bath (37 °C).

5.3.2. Run the double digested products on a 1% agarose gel and then cut the bands under UV light. In the case of luciferase vectors, before running on a gel, react double digested vectors with 10 U of alkaline phosphatases for another 1 h to prevent a recircularization during the ligation step.

5.3.3. Purify the double digested PCR products and luciferase vectors from the excised bands.

5.4. Ligation of PCR products into the luciferase vectors

5.4.1. Make 20 µL of ligation reaction mixtures including the DNA ligase (**Table 4**).

NOTE: The molar ratio of PCR product (insert) to luciferase vector can be 3:1. 1:1 or 2:1.

5.4.2. Briefly centrifuge the tube for 10-15 s and incubate at 16 °C overnight using a thermal cycler.

NOTE: Alternatively, the tube can be incubated at 4 °C for 2-3 days for the ligation. In this step, the PCR insert will be cloned into the region positioned downstream of a renilla reporter gene (**Figure 5B**). Binding of miRNAs into the cloned 3' UTR of a gene can decrease in the renilla activity. Firefly luciferase is for the normalization of renilla expression levels.

5.5. Transformation and colony PCR

5.5.1. Add the ligation mixtures (3-5 µL) into the tube containing competent cells. Gently tap the tube and keep it on ice (20 min).

NOTE: Unfreeze competent cells on ice before adding the ligation mixtures.

5.5.2. Quickly and gently transfer the tube to a heat block. Following a heat-shock (42 °C for 30 s-1 min), place the tube on ice for 20 min.

5.5.3. Spread competent cells on the Luria-Bertani (LB) agar plate. Grow competent cells in an incubator (37 °C) overnight.

NOTE: Ampicillin (50-100 µg/mL) is contained in the agar plate.

5.5.4. Pick an individual colony and resuspend *E. coli* in one of the 8-strip tubes containing ultrapure water. Repeat this step to resuspend *E. coli* from randomly selected 4-8 colonies (**Figure 5C**).

5.5.5. Transfer 25 µL of *E. coli* suspension into another set of 8-strip tubes. Now, there are 2 sets of tubes of *E. coli* suspension.

NOTE: One tube is for colony PCR and another one is for inoculation. *E. coli* suspension for inoculation can be temporarily stored at 4 °C (**Figure 5C**).

5.5.6. Perform the colony PCR using *E. coli* suspension. This step is to determine if the colonies contain an insert. Select the best colonies to inoculate and isolate luciferase vectors harboring 3' UTR of a gene (**Figure 5C**).

NOTE: Repeat step 5.1-5.5 for each 3' UTR of selected genes. Follow the condition of PCR reaction shown in **Table 2** by replacing the genomic DNA with *E. coli* suspension.

5.6. Luciferase assay

5.6.1. Prepare a 24-well plate. Use 1-2 x 10^4 cells in 500 µL cell culture media for each well. Do not use cell culture media containing P/S for the transfection because using P/S can reduce the transfection efficiency.

5.6.2. Transfect 50 ng of luciferase vectors into the cells with control mimic or a specific miRNA mimic using a transfection reagent (**Figure 5D**). If screening the effects of a specific miRNA mimic at more than one concentration, keep the total amount of oligos same in each well (see step 2).

5.6.3. Wash the inside of wells twice using phosphate buffered saline (PBS) on the next day.

5.6.4. Apply 200 µL of lysis reagent into the wells and sufficiently carry out cell lysis before measuring the luciferase activity.

NOTE: Keep the plate on a shaking plate at least 15 min.

5.6.5. Transfer 5-10 µL of cell lysate into the new tube and add 100 µL of reagent I. Immediately mix the solution by pipetting and read the firefly luciferase activity using a luminometer.

NOTE: Read the firefly luciferase activity for 10-15 s.

5.6.6. Add 100 µL of reagent II in the same tube, and then mix by pipetting twice. Read the renilla luciferase activity for 10-15 s using a luminometer. Repeat step 5.6.5 and 5.6.6 for each sample.

5.6.7. Calculate the ratio of renilla to firefly (**Figure 5E**).

NOTE: The activity of firefly represents the transfection efficiency of luciferase constructs into the cells.

REPRESENTATIVE RESULTS

Successful and accurate confirmation of miRNA levels is important for the interpretation of data by which the classification of miRNAs is possible based on the anticipated roles of miRNAs in the development and progression of a disease. The levels of miRNA-107 and miRNA-301 were measured in three pancreas cell lines using the probe-based quantitative PCR. The synthesis of cDNAs of both a specific miRNA and a reference gene in the same reaction can increase the reproducibility of data. PANC-1 and CAPAN-1 are human pancreatic ductal adenocarcinoma cell lines, while HPNE is an immortalized pancreas duct cell line transduced with a retroviral expression vector harboring the human telomerase reverse transcriptase (hTERT) gene. miRNA-107 was significantly reduced in PANC-1 and CAPAN-1 cells compared with HPNE cells (**Figure 2C**). The levels of miRNA-301 were significantly up-regulated in PANC-1 and CAPAN-1 cells compared with HPNE cells. These results are in accordance with previous reports that miRNA-107 is epigenetically inactivated in pancreatic cancer cells and that miRNA-301 levels are higher in pancreatic ductal adenocarcinoma cells than normal pancreatic ductal cells[16,17].

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reflects cell metabolic activities. This feature has a significantly higher opportunity to acquire the lack of correlation between the MTT assay and the total cell number since the assay conditions including a kind of treatment reagents can severely affect the enzymatic reduction of tetrazolium[18,19]. To overcome this limitation, the sulforhodamine B (SRB) assay was applied to measure the effects of miRNA-107 on cell proliferation to identify the potential biological functions of miRNA-107. The amount of bound SRB dye in the fixed cells can be used as a surrogate of the change in total number of cells. The SRB assay in this study clearly demonstrates that the proliferation of PANC-1 cells decreased following a miRNA-107 mimic transfection (**Figure 3**). The miRNA-107 concentration that caused an inhibition of 50% cell viability (IC_{50}) was determined by the generation of a dose-response curve. In addition, the application of Equation 2 is beneficial to calculate all possible inhibitory concentrations (ICx) for precisely evaluating the effects of miRNA-

107 (**Figure 4**).

Examination of the correlation between miRNAs and mRNAs levels is an effective way for the miRNA target identification because miRNAs can regulate target gene levels via the degradation of mRNAs[2,20]. However, since miRNAs also act at the translational level without affecting the processes of mRNA degradation, the experimental validation of miRNA-target gene interactions using the luciferase assay is an essential step. The main advantage of a luciferase assay is that this assay can rule out the changes of mRNA levels regulated by the degradation of mRNAs[21]. Therefore, the cloning of 3' UTR of predicted target genes is an important step to identify real target genes of a miRNA of interest in conjunction with real-time PCR and western blot experiments. An efficient utilization of the dual reporter vectors allows to acquire unambiguous experimental data since the measurement of control reporter levels can reduce the experimental variations such as the number of cells. Normalization processes of the luciferase assay data acquired from vectors containing 3' UTR of a synuclein gamma (SNCG) gene is shown in **Figure 5E**. In addition, the screening of 11 potential predicted targets of miRNA-107 clearly shows that only SNCG, a positive regulator of tumor cell growth[22,23], directly interacts with miRNA-107 in PANC-1 cells (**Figure 6**). This finding indicates that miRNA-107 can negatively regulate the proliferation of PANC-1 cells by modulating the SNCG expression.

FIGURE AND TABLE LEGENDS

Figure 1: Experimental design for miRNA target identification. This diagram shows the flow of an experimental design which helps to identify the target of a miRNA. **(A)** The analysis of mature miRNA expression levels and the selection of a miRNA of interest. **(B)** Three experiments, such as the miRNA mimic transfection, SRB assay, and generation of a dose-response curve can be conducted for the functional study of selected miRNAs. **(C)** To narrow down candidate target genes of a miRNA of interest, it is beneficial to check the information and function of predicted target genes in target prediction programs, Pubmed, and GeneCard. **(D)** After detecting some potential candidate target genes of a miRNA of interest, it is feasible to conduct practical experiments such as the cloning of 3' UTR of mRNAs, luciferase assay, real-time PCR, and western blot to finally prove the direct target genes of a miRNA of interest.

Figure 2: Mature miRNA expression analysis. (A) Add total RNA from each cell line (PANC-1, CAPAN-1, and HPNE), DNase I mixtures, and ultrapure water into labeled strip-tubes. Total volume in each tube is 18 µL (PANC-1 and CAPAN-1 are pancreatic cancer cell lines, while HPNE is a normal pancreatic duct cell line). **(B)** Transfer 7.1 µL of DNase I treated mixtures into 2 sets of new strip-tubes, and 1.5 µL of antisense primers for a GAPDH gene is also added in each tube. Incubate PCR strip-tubes at indicated reaction conditions. Next, add RT enzyme mixtures and 5X RT primers for a specific miRNA (miRNA-107 or miRNA-301) into its designated tubes, and then re-incubate the tubes at the indicated conditions. Single-stranded cDNAs for a specific miRNA and GAPDH are generated. **(C)** Mature miRNA-107 and miRNA-301 levels were determined by the probe-based real-time PCR using total RNA isolated from PANC-1, CAPAN-1, and HPNE cells.

Figure 3: Mimic transfection and the SRB Assay. (A) Top panel shows the dilution of miRNA control mimic and miRNA-107 mimic to prepare transfection mixtures. The range of final concentrations of miRNA-107 mimic are 0 nM, 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, and 100 nM. Total volume in each column is for the transfection of cells in one well of a 96-well plate. Bottom panel shows an example of scaling up the transfection mixtures to prepare enough amount of mixtures for the transfection of the cells in 4 wells at an indicated concentration. Next, add 50 µL of mixtures into each well by following the suggested format for transfecting miRNA control mimic with miRNA-107 mimic. This plate was used for the SRB assay, which includes cell fixation, cell staining, and absorbance measurement. **(B)** Actual image of the 96-well plate showing the SRB stained cells. This image clearly shows that the number of PANC-1 cells decreases as the concentration of miRNA-107 mimic increases.

Figure 4: Generation of a dose-response curve. (A) Representative dose-response curve of miRNA-107 mimic transfected PANC-1 cells. Transfected cells were incubated continuously for 96 h. Parameters procured from the dose-response curve are also shown. **(B)** The equation, variables, initial parameters, and constraints, along with the descriptions of definitions and values. **(C)** Insert the definitions and values into the corresponding panels in the software. The "f" indicates the % cell viability (for example, the value of "f" is "90" and "10" at IC_{10} and IC_{90}, respectively). The y0 value is 100 and indicates the 100% cell viability of miRNA control mimic transfected cells. The "n" indicates the Hill-type coefficient (the slope of a plot). The "k" indicates the concentration of miRNA-107 mimic that produces a 50% of the miRNA-107 mimic's maximum effect (IC_{50}). The "R" indicates the residual unaffected fraction (the resistance fraction). **(D)** This panel shows how to calculate adjusted ICx values based on the parameters (n, k, and R) acquired from Equation 1. The percentage (%) cell viability in the panel represents "f" in Equation 1 and calculated by subtracting the x value of each ICx from y0 (100). Equation 2 in the formula bar of the spreadsheet is indicated as "=((100-D3)/(D3-$B$5))^(1/$B$3)*$B$4)" for the calculation of adjusted IC_{10} value. Apply Equation 2 to other cells for calculating other ICx values by pressing the left mouse button on the selected cell (red color) and dragging down to the cell of IC_{90} value.

Figure 5: Verification of the direct target gene of a miRNA of interest. Experiments begin with designing primers for the cloning of 3' UTR. Primers are used for the gradient PCR. **(A)** The best annealing temperature can be selected among the six indicated annealing temperatures to amplify 3' UTR of a gene. Next, double digestion is performed with restriction enzymes and PCR products are ligated into luciferase vectors. **(B)** Luciferase vectors containing 2 reporter genes, firefly and renilla luciferase, can be used to screen the interactions of miRNAs with 3' UTR of mRNAs. PCR inserts are cloned into a region placed downstream of the renilla reporter gene. Ligated products were transformed into competent cells and grew cells on the LB agar plate. **(C)** Individual colonies (#1 to #6) were picked up and resuspended in 50 µL of ultrapure water. The *E. coli* suspension was used for the colony PCR and inoculation. Colony PCR is a convenient tool to select the best colonies for the inoculation and isolation of luciferase vectors harboring 3' UTR of a gene. **(D)** For the luciferase assay, miRNA control mimic or miRNA-107 mimic was transfected into PANC-1 cells with luciferase constructs using a 24-well plate. (**E**) This panel demonstrates the representative raw data and calculation of the ratio of renilla to firefly after executing the luciferase assay for the validation of a SNCG gene as a miRNA-107 target.

Figure 6: Screening of predicted target genes of miRNA-107. Screening of interactions between miRNA-107 and 3' UTR of predicted targets was performed in PANC-1 cells using the luciferase constructs. Based on the negative effects of miRNA-107 on cell proliferation, potential candidate genes were determined for the cloning and screening assays. miRNA control mimic or miRNA-107 mimic was transfected into PANC-1 cells with luciferase constructs containing 3' UTR of each selected gene for 24 h. The ratio of renilla to firefly was calculated and normalized based on the measured levels of both luciferases in PANC-1 cells.

Table 1: Conditions used for a specific miRNA and GAPDH detection by the real-time PCR in this study.

Table 2: Composition of PCR reaction mixtures for the amplification of 3' UTR in this study.

Table 3: Conditions for the double digestion of PCR products and luciferase vectors using XhoI (or AsiSI) and NotI enzymes in this study.

Table 4: Ligation reactions of double digested PCR products and luciferase vectors with the DNA ligase in this study.

Supplementary Figure 1: The derivation of Equation 2 from Equation 1. Equation 2 is derived from Equation 1 for the calculation of adjusted ICx values.

Supplementary Figure 2: Primer information.

DISCUSSION

Strategies for the determination of bona fide miRNA targets with the functions of a miRNA of interest are indispensable for the understanding of multiple roles of miRNAs. Identification of miRNA target genes can be a guideline for interpreting the cell signaling events modulated by miRNAs in a cell. An unveiling of functionally important target genes of miRNAs can provide the fundamental knowledge to develop a miRNA-based therapy in cancer.

Several methods such as microarrays, small RNA library sequencing, deep sequencing, reverse transcriptase in situ PCR, and northern blotting can be applied to explore miRNA expression levels using total RNA isolated from cell lines and tissues[24-26]. High throughput profiling of miRNAs can provide valuable insight into the genetic underpinnings of cancer development. The probe-based miRNA assay is often used to validate profiling data and is also suitable to screen a few miRNAs of interest. However, the measurement of mature miRNA levels using the probe-based real-time PCR is limited to determine whether mature miRNAs are regulated at the transcription steps or through the regulation of maturation. Dye-based real-time PCR and northern blotting have been introduced to measure miRNA precursor levels[27,28]. Measurement of miRNA precursors together with mature miRNA levels can further provide the information on how mature miRNA levels are regulated. Furthermore, a comprehensive understanding of the regulation of miRNA levels is indispensable for the development of miRNA-based therapeutic approaches.

Cell proliferation assays such as the tetrazolium-based MTT assay are applicable to screen the effects of treatment reagents such as miRNA mimics. Since the MTT assay reflects the cell metabolic activities based on the tetrazolium reduction by cellular oxidoreductases, it is possible to observe the lack of correlation between the MTT assay and the total cell number[19]. Alternatively, the SRB assay is available as the most reproducible cell enumeration assay[18,19]. Measurement of the amount of SRB dye bound to trichloroacetic acid fixed proteins can represent the total cell number[29]. In addition, the SRB assay in this protocol can be applied to screen multiple miRNA mimics as well as anti-cancer drugs using 384-well plates. However, the SRB assay has limitations such as manual screening. In addition, this assay is not available to non-adherent cells. Since miRNAs also play a critical role in hematologic malignancy such as lymphoma and myeloma[30], efficient monitoring of cell proliferation is required to unravel the functions of miRNAs. Carboxyfluorescein succinimidyl ester (CFSE) can intracellularly label the non-adherent cells. CFSE is used to monitor the generation of proliferating cells by flow cytometry[31]. In addition, miRNAs can affect invasion, metastasis, and programmed cell death. Therefore, other experimental techniques combining with this protocol will be more practical for the proper understanding of miRNA functions, which ultimately contribute to identifying multitudinous biologically relevant targets of miRNAs.

Calculation of the half maximal inhibitory concentration (IC_{50}) is an important method not only for the miRNA studies but also for the efficacy evaluation of other anti-cancer drugs. IC_{50} values can be used to compare the potential effects of several miRNAs or anti-cancer drugs on cell proliferation. It has been demonstrated that the combination of a miRNA-based therapy with anti-cancer drugs can provide an exceptional opportunity to improve the anti-cancer drug's efficacy. Furthermore, the combination of miRNAs with anti-cancer drugs can be a novel approach to overcome chemoresistance[32,33]. For the evaluation of combination efficiency, it is advantageous to calculate adjusted ICx values based on our protocol for the assessment of combination index (CI) which allows the quantitative estimation of synergism or antagonism[33,34].

Intracellular signaling networks can be widely disorganized by anomalously expressed miRNAs in numerous diseases including cancers. However, signaling networks convolutedly affected by miRNAs are still mostly unknown since the small proportion of target genes have been experimentally validated, and target genes are also regulated via non-canonically reacting with miRNAs[35]. Nonetheless, our strategy and protocol are reliable methods for deciphering the cellular mechanisms of miRNAs. In addition, our protocol can be further extended to implement and evaluate the combination of miRNAs and other anti-cancer drugs.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

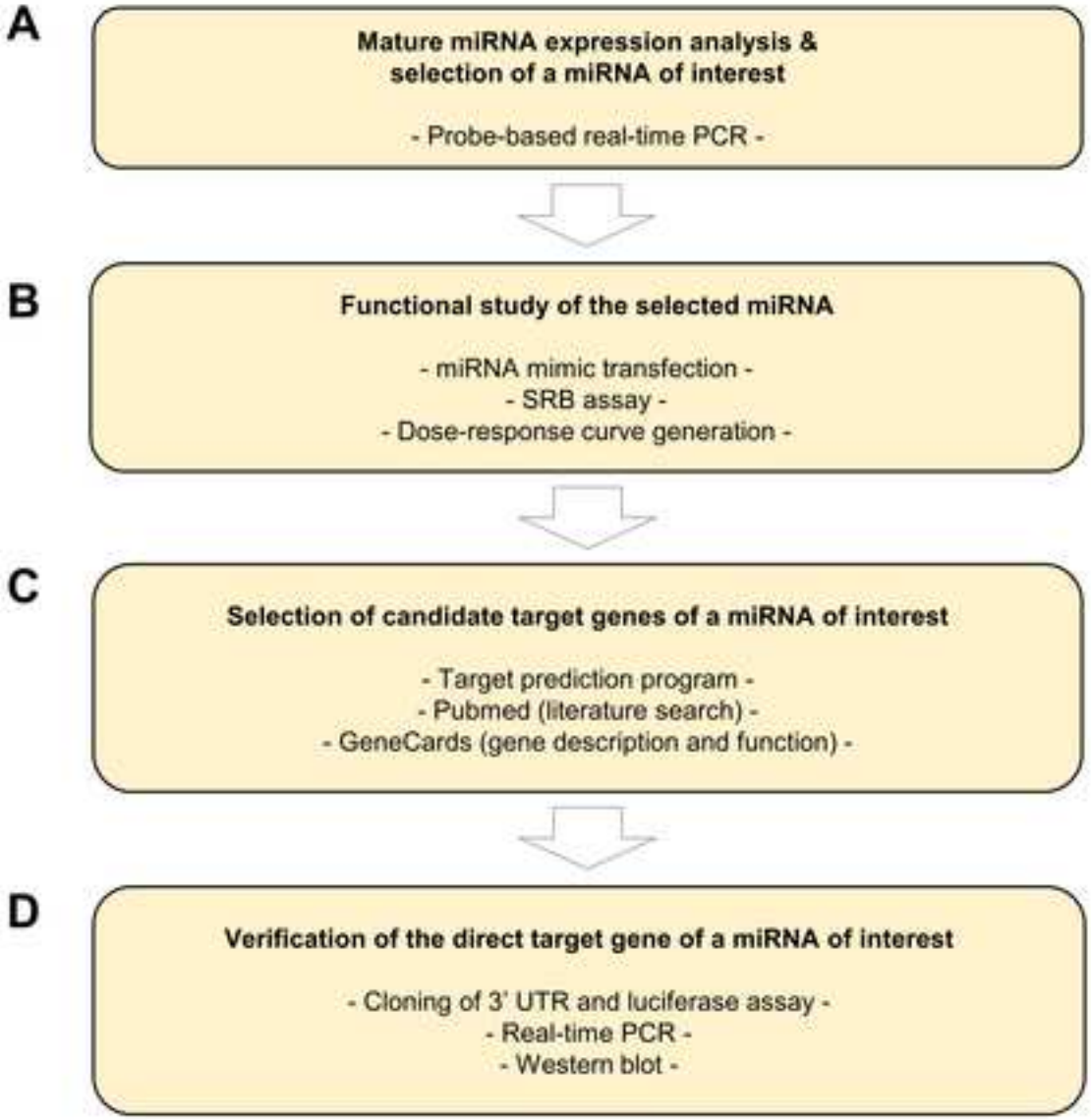


Figure 2

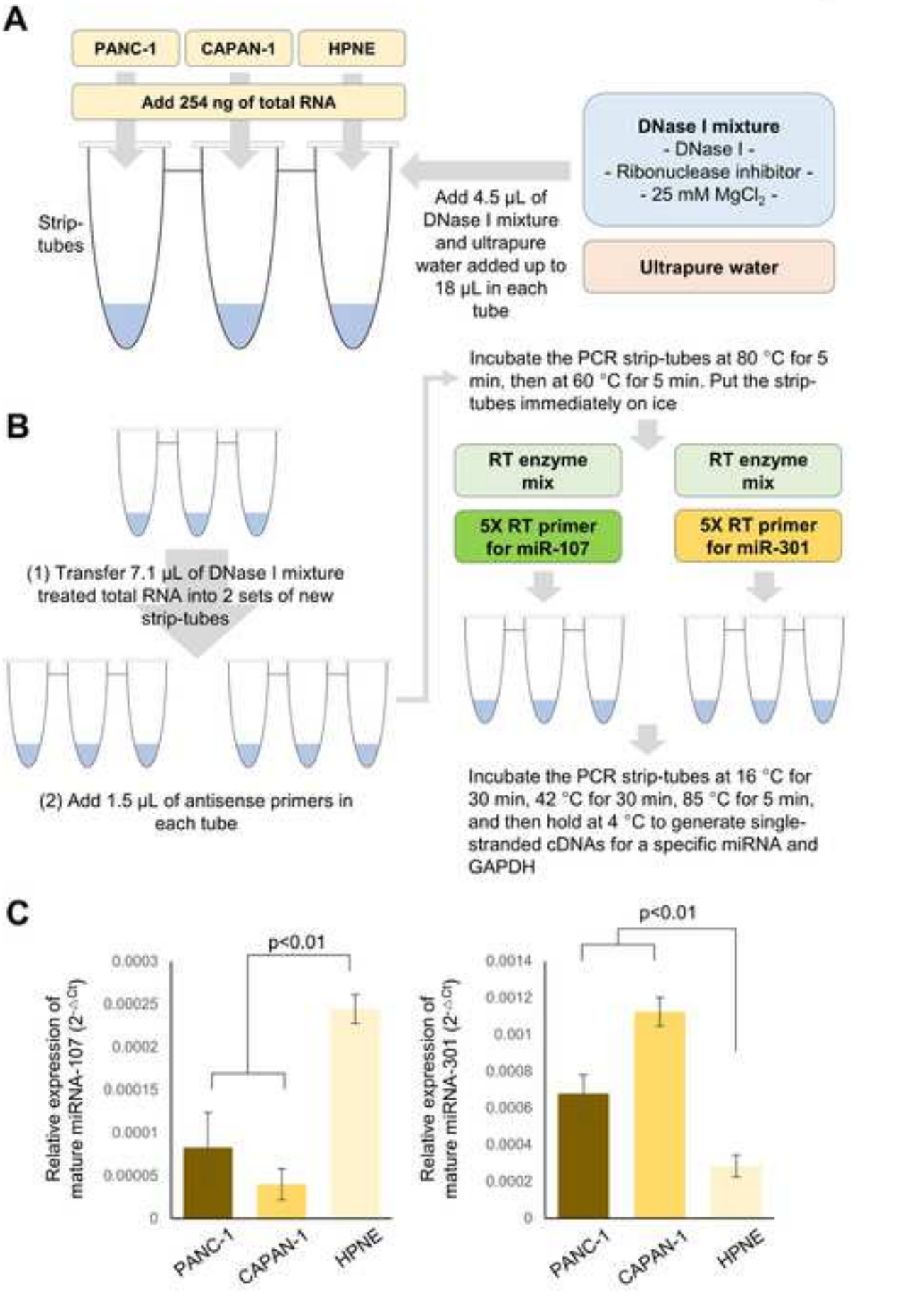


Figure 3 Click here to access/download;Figure;Figure 3.TIF

Figure 3

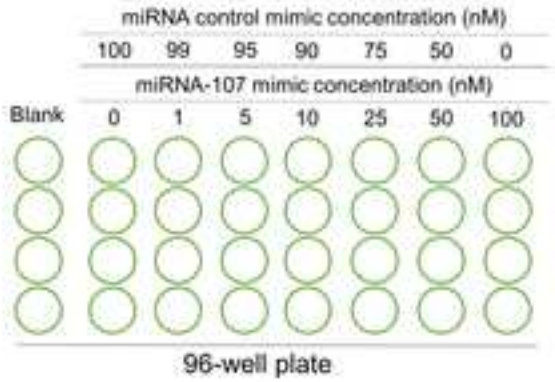
A

Final miRNA-107 mimic concentration	0 nM	1 nM	5 nM	10 nM	25 nM	50 nM	100 nM
Reduced-serum media (μL)	48.5	48.5	48.5	48.5	48.5	48.5	48.5
miRNA control mimic (stock 25 μM)							
Stock dilution fold (concentration)	0 (25 μM)	1.01 (24.75 μM)	1.05 (23.75 μM)	1.11 (22.5 μM)	1.33 (18.75 μM)	2 (12.5 μM)	
Control mimic or ultrapure water (μL)	0.6	0.6	0.6	0.6	0.6	0.6	0.6 (water)
miRNA-107 mimic (stock 25 μM)							
Stock dilution fold (concentration)		100 (0.25 μM)	20 (1.25 μM)	10 (2.5 μM)	4 (6.25 μM)	2 (12.5 μM)	0 (25 μM)
miRNA-107 mimic or ultrapure water (μL)	0.6 (water)	0.6	0.6	0.6	0.6	0.6	0.6
Transfection reagent (μL)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total (μL)/well	50	50	50	50	50	50	50

⇩ Scale up each volume 5 times to get the enough amount of mixtures for 4 wells per concentration

Final miRNA-107 mimic concentration	0 nM	1 nM	5 nM	10 nM	25 nM	50 nM	100 nM
Reduced-serum media (μL)	242.5	242.5	242.5	242.5	242.5	242.5	242.5
miRNA control mimic (stock 25 μM)							
Stock dilution fold (concentration)	0 (25 μM)	1.01 (24.75 μM)	1.05 (23.75 μM)	1.11 (22.5 μM)	1.33 (18.75 μM)	2 (12.5 μM)	
Control mimic or ultrapure water (μL)	3	3	3	3	3	3	3 (water)
miRNA-107 mimic (stock 25 μM)							
Stock dilution fold (concentration)		100 (0.25 μM)	20 (1.25 μM)	10 (2.5 μM)	4 (6.25 μM)	2 (12.5 μM)	0 (25 μM)
miRNA-107 mimic or ultrapure water (μL)	3 (water)	3	3	3	3	3	3
Transfection reagent (μL)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Total (μL)/well	250	250	250	250	250	250	250

⇩ Add 50 μL of mixtures into each well



B

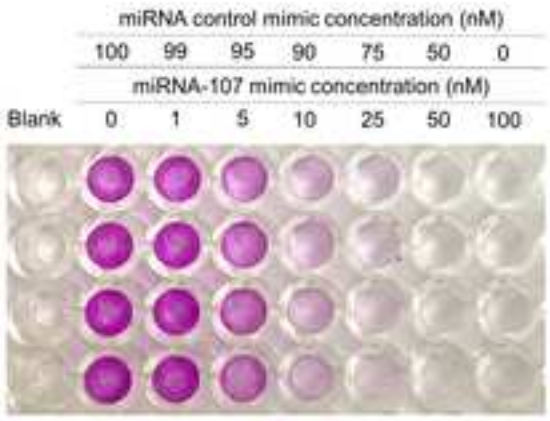


Figure 4

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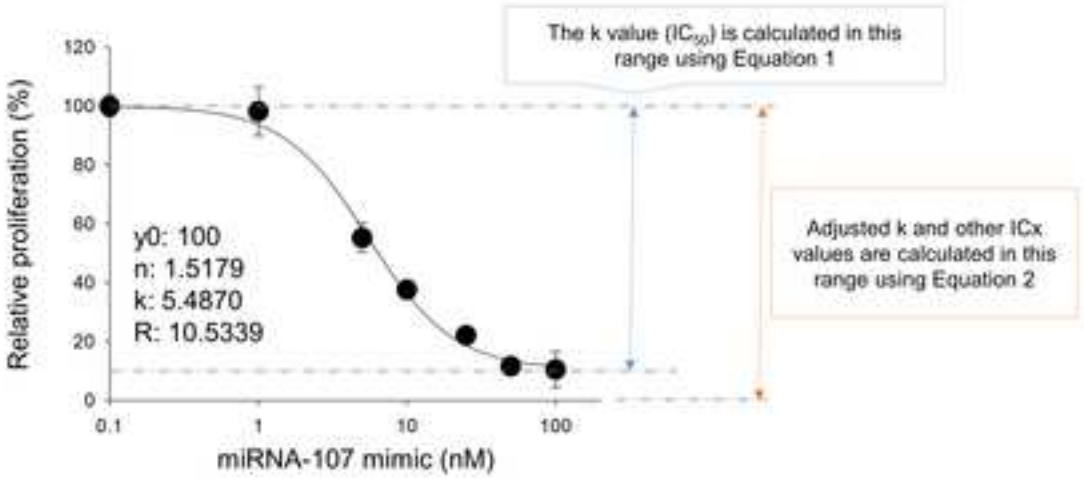
Figure 4

A



B

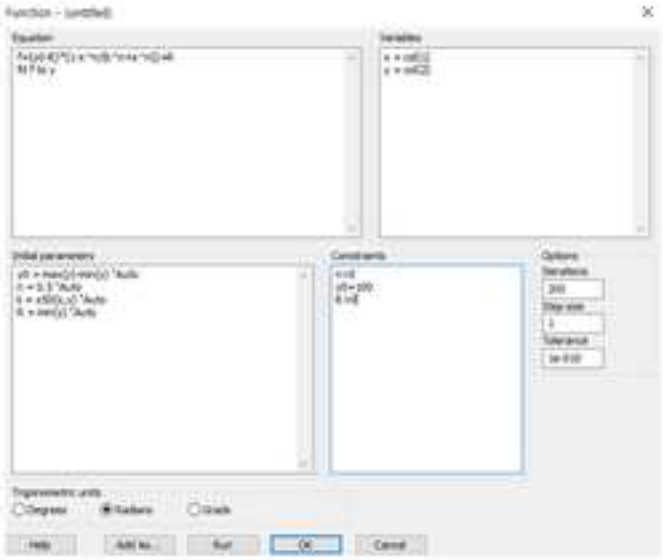
Equation
f=(y0-R)*(1-x^n/(k^n+x^n))+R
fit f to y

Variables
x = col(1)
y = col(2)

Initial parameters
y0 = max(y)-min(y) "Auto
n = 0.5 "Auto
k = x50(x,y) "Auto
R = min(y) "Auto

Constraints
n>0
y0=100
R>0

C



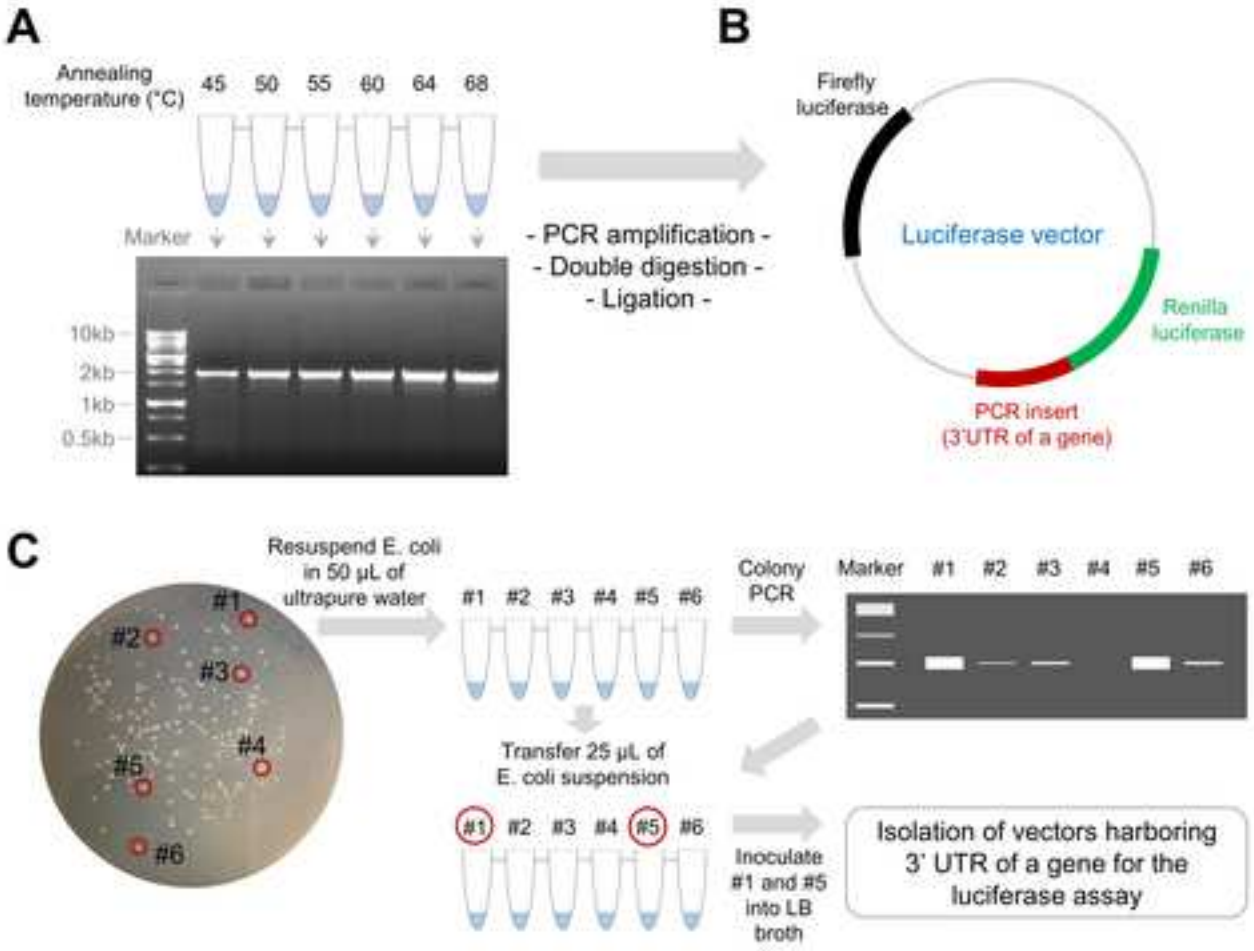
D

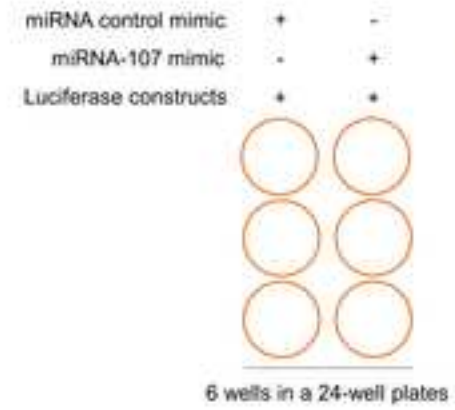
	A	B	C	D	E
1	Calculation of adjusted values of ICx				
2	Parameters from Figure 4C		ICx	% cell viability	ICx values
3	n	1.5179	IC10	90	1.40052443
4	k	5.487	IC20	80	2.415966226
5	R	10.5339	IC30	70	3.496006733
6			IC40	60	4.770476207
7			IC50	50	6.412455946
8			IC60	40	8.765824238
9			IC70	30	12.7500332
10			IC80	20	22.38688656
11			IC90	10	#NUM!

=((100-D3)/(D3-$B$5))^(1/$B$3)*$B$4

Apply the formula by dragging down

Figure 5





E

Transfection condition	Measurement		Ratio (R/F)	Normalized (%)
	Firefly	Renilla		
Control mimic + constructs	105	3321	31.6	100±6.4
	98	3413	34.8	
	110	3401	30.9	
miRNA-107 mimic + constructs	92	2077	22.6	65.9±3.2
	114	2364	20.7	
	101	2109	20.9	

Figure 6

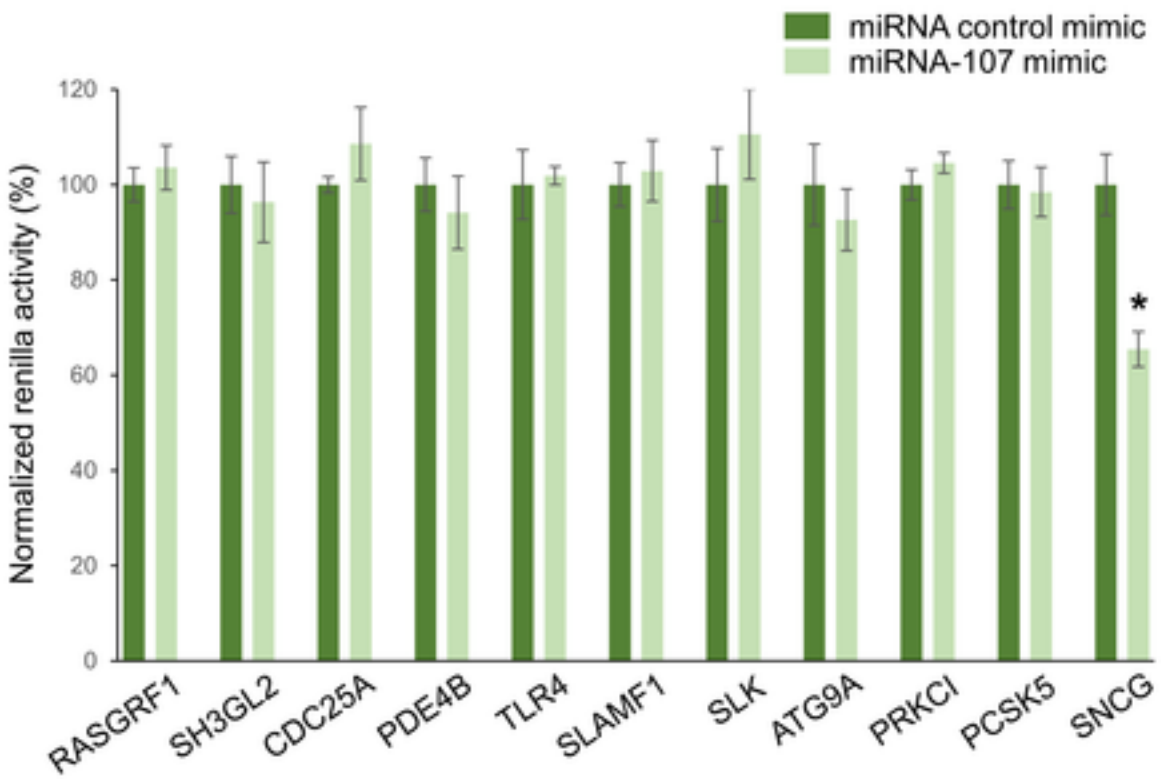


Table 1

Detection	miRNA	GAPDH
Components		
Master mix for probe-based real-time PCR (2X)	10 µL	–
Master mix for dye-based real-time PCR (2X)	–	10 µL
Probe mixture (5X)	4 µL	–
GAPDH primers (1 µM each)	–	4 µL
Diluted cDNA (1:49)	6 µL	6 µL
Total volume	20 µL	20 µL

Table 2

Components	1X reaction	7X reaction
5X buffer	5 µL	35 µL
dNTP mixture (2.5 mM each)	2 µL	14 µL
Primers (10 µM each)	1 µL	7 µL
Genomic DNA (2 ng/µL)	16.5 µL	115.5 µL
Polymerase	0.5 µL	3.5 µL
Total volume	25 µL	175 µL

Table 3

Components	1X reaction
10X buffer	5 µL
PCR products or vectors	PCR products: 25 µL Vectors: 1-2 µg
XhoI (or AsiSI) restriction enzyme	2 µL
NotI restriction enzyme	2 µL
Ultrapure water	x µL
Total volume	50 µL

Table 4

Components	1X reaction
10X buffer	2 µL
Vectors (double digested)	50 ng
PCR products (insert)	x µL
Ligase	200 U
Ultrapure water	y µL
Total volume	20 µL

Table of Materials

Click here to access/download;Table of Materials;Materials and Equipments_JK Park.xlsx

Name of Material/ Equipment	Company	Catalog Number
15 mL conical tube	SPL Life Sciences	50015
24-well plate	Thermo Scientific	142475
50 mL conical tube	SPL Life Sciences	50050
6-well plate	Falcon	353046
6X DNA loading dye	Real Biotech Corporation	RD006
8-cap strip	Applied Biosystems	N8010535
8-tube strip	Applied Biosystems	N8010580
96-well plate	Falcon	353072
Acetic acid	Sigma	A6283-1L
Agarose A	Bio Basic	D0012
Alkaline phosphatase	New England Biolabs	M0290S
Ampicillin	Bio basic Canada Inc	AB0028
AriaMx 96 tube strips	Agilent Technologies	401493
AriaMx real-time PCR system	Agilent Technologies	G8830A
AsiSI	New England Biolabs	R0630
CAPAN-1 cells	ATCC	HTB-79
Cell culture hood	Labtech	
Counting chambers with V-slash	Paul Marienfeld	650010
CutSmart buffer	New England Biolabs	B7204S
DMEM	Gibco	11965-092
DNA gel extraction kit	Bionics	DN30200
DNA ladder	NIPPON Genetics EUROPE	MWD1
DNase I	Invitrogen	18068015
Dual-luciferase reporter assay system	Promega	E1910
Fetal bovine serum	Gibco	26140-079
HIT competent cells	Real Biotech Corporation(RBC)	RH617
HPNE cells	ATCC	CRL-4023
LB agar broth	Bio Basic	SD7003
Lipofectamine 2000	Invitrogen	11668-027
Lipofectamine RNAiMax	Invitrogen	13778-075
Luminometer	Promega	
Microcentrifuge tube	Eppendorf	22431021
Microplate reader	TECAN	
miRNA control mimic	Ambion	4464058
miRNA-107 mimic	Ambion	4464066
miRNeasy Mini Kit	Qiagen	217004
Mupid-2plus (electrophoresis system)	TaKaRa	
NotI	New England Biolabs	R3189
Oligo explorer program	GeneLink	
Optical tube strip caps (8X Strip)	Agilent Technologies	401425
Opti-MEM	Gibco	31985-070
PANC-1 cells	ATCC	CRL-1469
Penicillin/streptomycin	Gibco	15140-122
Phosphate buffer saline	Gibco	14040117
Plasmid DNA miniprep S& V kit	Bionics	DN10200
PrimeSTAR GXL DNA polymerase	TaKaRa	R050A

Shaker	TECAN	
Shaking incubator	Labtech	
Sigmaplot 14 software	Systat Software Inc	
Sulforhodamine B powder	Sigma	S1402-5G
SYBR green master mix	Smobio	TQ12001805401-3
T4 DNA ligase	TaKaRa	2011A
TaqMan master mix	Applied Biosystems	4324018
TaqMan microRNA assay (hsa-miR-107)	Applied Biosystems	4427975
TaqMan microRNA assay (hsa-miR-301)	Applied Biosystems	4427975
TaqMan miR RT kit	Applied Biosystems	4366597
Thermo CO_2 incubator (BB15)	ThermoFisher Scientific	
Trichloroacetic acid	Sigma	91228-100G
Trizma base	Sigma	T4661-100G
Ultrapure water	Invitrogen	10977-015
Veriti 96 well thermal cycler	Applied Biosystems	
XhoI	New England Biolabs	R0146

Comments/Description

1 mL
For cDNA synthesis
For cDNA synthesis

1 L
500 g
10,000 U/mL
25 g
For real time PCR
qPCR amplification, detection, and data analysis
10,000 units/mL

Model: LCB-1203B-A2
Cells counter
10X concentration
500 mL
200 prep
1 Kb ladder
100 units

100 assays
500 mL
Competent cells

250 g
0.75 mL
0.75 mL
Model: E5311

Infinite F50
5 nmole
5 nmole
50 prep
Model: AD110
20,000 units/mL
For primer design
For real time PCR
500 Ml

100 mL
1000 mL
200 prep
250 units

Shaking platform
Model: LSI-3016A
For dose-response curve generation
5 g
Binding fluorescent dye for dsDNA
25,000 U
200 reactions, no AmpErase UNG

Assay ID: 000443 (50RT, 150 PCR rxns)

Assay ID: 000528 (50RT, 150 PCR rxns)
1000 reactions
37 °C and 5% CO_2 incubation
100 g
100 g
500 mL
For amplification of DNA (or cDNA)
20,000 units/mL

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Author(s):	Hyun Ah Seo, Cho Yean Hwang, Sokviseth Moeng, and Jong Kook Park

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CORRESPONDING AUTHOR

Name:	Jong Kook Park
Department:	Department of Biomedical Science
Institution:	Hallym University
Title:	Professor
Signature:	Jong Kook Park
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March 05, 2019

Bing Wu, Ph.D.

Review Editor

Journal of Visualized Experiments

Re: JoVE manuscript #59628_R1

Dear Dr. Bing Wu

We appreciate the helpful editorial comments. We made all of the changes as requested by the editor. Our responses to comments are detailed below.

Editorial comments:

The manuscript has been modified and the updated manuscript, **59628_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- Thank you very much to give us a chance to carefully proof-read the manuscript again. We made the all necessary corrections throughout the manuscript. In addition, we switched the order of subpanels in Fig. 4 (its legend as well) and protocol descriptions for the efficient filming.

2. Please do not abbreviate journal titles for all references.

- In the revised version of manuscript, we changed all journal titles to their full names.

3. Please define all abbreviations before use, e.g., FBS, etc.

- We checked the manuscript carefully and made the all necessary corrections.

4. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include lipofectamine, RNAiMAX, etc.
- Thank you very much for this information. We changed all commercial (commercial-like) terms to general terms. For example, Taqman and RNAiMAX was changed to probe-based and

transfection reagent, respectively. The change of commercial terms was down in the main manuscript, figures with their legends, and tables.

5. Step 2.2: What's the condition for transfection? Please add more details.
- We revised step 2.2 and also add NOTE to explain about the transfection conditions.

6. 4.3.8: Please split this long step into more sub-steps.
- In this revised manuscript, we changed the order of step 4 for the efficient filming. In addition, step 4.3.8 (4.8 in the revised version) was divided into 2 steps and made the new step (step 4.9).

7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next.

- We highlighted steps again for the proper filming process. Highlighted steps are from step 2 to step 5.6.6 and we confirm that it does not exceed 2.75 pages.

Since we have complied with all of the suggestions made by the Editor, we hope that you will now deem this paper acceptable for the filming and publication in **Journal of Visualized Experiments**, and are eager to see it in print.

Sincerely yours,

Jong Kook Park, PhD

Department of Biomedical Science and Research Institute for Bioscience & Biotechnology, Hallym University, Chuncheon 24252, Republic of Korea

Phone: 82-33-248-2114

Fax: 82-33-256-3420

E-mail: jkp555@hallym.ac.kr

Supplementary Figure 1: The derivation of equation 2 from the equation 1. Equation 2 is derived from Equation 1 for the calculation of adjusted ICx values.

$$f = (y0 - R) \times \left(1 - \frac{x^n}{k^n + x^n}\right) + R$$

$$\downarrow$$

$$f - R = (y0 - R) \times \left(\frac{k^n + x^n}{k^n + x^n} - \frac{x^n}{k^n + x^n}\right)$$

$$\downarrow$$

$$f - R = (y0 - R) \times \left(\frac{k^n}{k^n + x^n}\right)$$

$$\downarrow$$

$$f - R = \frac{(y0 - R)\,(k^n)}{k^n + x^n}$$

$$\downarrow$$

$$(f - R) \times (k^n + x^n) = (y0 - R) \times k^n$$

$$\downarrow$$

$$f \cdot k^n + f \cdot x^n - R \cdot k^n - R \cdot x^n = y0 \cdot k^n - R \cdot k^n$$

$$\downarrow$$

$$f \cdot x^n - R \cdot x^n = y0 \cdot k^n - f \cdot k^n$$

$$\downarrow$$

$$x^n(f - R) = k^n(y0 - f)$$

$$\downarrow$$

$$\frac{x^n}{k^n} = \frac{y0 - f}{f - R}$$

$$\downarrow$$

$$\left(\frac{x}{k}\right)^n = \frac{y0 - f}{f - R}$$

$$\downarrow$$

$$\frac{x}{k} = \sqrt[n]{\frac{y0 - f}{f - R}}$$

$$\downarrow$$

$$x = k \times \sqrt[n]{\frac{y0 - f}{f - R}}$$

Supplementary Figure 2: Primer information

Genes	Direction	Sequences
RASGRF1	Sense	5' CTG CCT CGA GTC TCT CCG AAT AGA ACC AAA 3'
	Antisense	5' ATT AGC GGC CGC ATA ATT CAT TTA ATT CTA CTC TGT AAT GAG 3'
SH3GL2	Sense	5' CTG CCT CGA GGA TGT TAT GCT GGC TGG CT 3'
	Antisense	5' ATT AGC GGC CGC TTT GGT TCT GTC TTC AGC AAT ATT TAT TG 3'
CDC25A	Sense	5' CTG CCT CGA GAG TCG TCT GAA GAA GCT CTG 3'
	Antisense	5' ATT AGC GGC CGC TCC TTA AAT CCA TCA AGA ACT AGG 3'
PDE4B	Sense	5' CTG CCT CGA GGT TGG TTT CAT CGA CTA CAT TGT C 3'
	Antisense	5' ATT AGC GGC CGC TAA TCT AAT CAT GCT TTT CTT TAT TCA CCT 3'
TLR4	Sense	5' ATA TGC GAT CGC AGA GGA AAA ATA AAA ACC TCC T 3'
	Antisense	5' ATT AGC GGC CGC GAT ATT ATA AAA CTG CAT ATA TTT AAT GTA TAC 3'
SLAMF1	Sense	5' CTG CCT CGA GCA CCA GAG ACC AAC AAA GGG AC 3'
	Antisense	5' ATT AGC GGC CGC TTG CAA TTT CCT TGA TGG ACT GG CT 3'
SLK	Sense	5' CTG CCT CGA GCA AAG GGA AGC ATT CTG TG 3'
	Antisense	5' ATT AGC GGC CGC AAC ATT ATA CTG TCA TTT TTA TCA TAA CAA 3'
ATG9A	Sense	5' CTG CCT CGA GAC AAG GCT GAG CAG GGT T 3'
	Antisense	5' ATT AGC GGC CGC TCT GGT CAA ACT CCC TTT TTA TTA AGG 3'
PRKCI	Sense	5' CTG CCT CGA GTC CTC ATT TTT CAA CCA TGT ATT CTA C 3'
	Antisense	5' ATT AGC GGC CGC TCA ATG GGT ATT ATA CAT TTT ATT TAA AGA C 3'
PCSK5	Sense	5' CTG CCT CGA GAC CAA CAC CAC CAT TCC A 3'
	Antisense	5' ATT AGC GGC CGC TGG TCT TGA TTT AAT TTT ATT TCC AAT T 3'
SNCG	Sense	5' CTG CCT CGA GAG ACT AGA GGG CTA CAG G 3'
	Antisense	5' ATT AGC GGC CGC CAG GAG TGG GCT CAA GTT T 3'
GAPDH	Sense	5' GAA GGT GAA GGT CGG AGT C 3'
	Antisense	5' GAA GAT GGT GAT GGG ATT TC 3'