Quantitation of MicroRNA-29 and MicroRNA-122 Based on Real-Time PCR Assay

การวัดปริมาณไมโครอาร์เอ็นเอ 29 และไมโครอาร์เอ็นเอ 122 โดยใช้เทคนิคเรียลไทม์พีซีอาร์

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ABSTRACT

MicroRNAs (miRNAs) are small, noncoding RNAs approximately 22 nucleotides (nt) in length that regulate gene expression and involved in many cellular processes such as cell differentiation, cell proliferation, apoptosis and signaling pathway. Previous studies found that miR-29 family (a, b and c) and miR-122 have the dominant role to regulate genes in many liver diseases such as liver cancer, hepatitis and fatty liver. Therefore, the aim of this study was to develop a detection method based on real-time PCR assay for quantitation of miR-29 family and miR-122 which might be useful as a possible biomarker for diagnosis of non-alcoholic fatty liver disease (NAFLD). Positive controls of miR-29 family and miR-122 were generated by an *in vitro* transcription technique. The miRNAs were extracted by microRNA purification kit followed by polyuridylation, reverse transcription and detection by quantitative real-time PCR (qPCR). The thermal profile of qPCR was optimized for specificity of the assay. No cross detection or non-specific amplification of miRNAs was observed. The sensitivity of the assay was validated by 10-folds serial dilution of the positive controls ranging from 10^8-10 copies/ µl. Results revealed that the limit of detection for each miRNA was approximately 100 copies/ µl. In addition, the assay was evaluated for clinical application by using miRNAs extracted from serum of patients with NAFLD. Results showed that the assay can be applied to detect and quantify miR-29 family and miR-122 extracted from NAFLD patients. Furthermore, results implied that miR-29c and miR-122 might be served as potential biomarkers for NAFLD. In conclusion, the assays based on real-time PCR for quantitation of miR-29 family and miR-122 were successfully developed. Therefore, this assay might be useful and attractive for detection and quantitation of miR-29 family and miR-122 as a possible biomarker of NAFLD.

บทคัดย่อ

ไมโครอาร์เอ็นเอ คืออาร์เอ็นเอขนาดเล็ก ที่ไม่กำหนดการสร้างโปรตีน มีขนาดประมาณ 22 นิวคลีโอไทด์ มีบทบาทในการควบคุมการแสดงออกของยีน และเกี่ยวข้องกับกระบวนการต่างๆ ของเซลล์ ได้แก่ การเปลี่ยนแปลงไปทำหน้าที่เฉพาะอย่าง การเพิ่มจำนวน การตายและการส่งสัญญาณภายในเซลล์ ซึ่งในงานวิจัยก่อนหน้านี้พบว่า ไมโครอาร์เอ็นเอ 29 (เอ บี และซี) และไมโครอาร์เอ็นเอ 122 มีบทบาทสำคัญ ในการควบคุมยีนที่เกี่ยวข้องกับโรคตับต่างๆ เช่น มะเร็งตับ ตับอักเสบ และไขมันในตับ เป็นต้น ดังนั้นวัตถุประสงค์ของการศึกษานี้คือ พัฒนาเทคนิคในการตรวจหา และวัดปริมาณไมโครอาร์เอ็นเอ 29 และไมโครอาร์เอ็นเอ 122 เพื่อใช้เป็นตัวชี้วัดทางชีวภาพสำหรับการตรวจวินิจฉัยโรคตับ

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คั่งไขมัน โดยใช้เทคนิคเรียลไทม์พีซีอาร์ ซึ่งขั้นตอนการสร้าง positive control จะใช้เทคนิคการสร้างอาร์เอ็นเอในหลอดทดลอง (*in vitro* transcription) แล้วจึงสกัดไมโครอาร์เอ็นเอ ตามด้วยขั้นตอน Polyuridylation และ Reverse transcription จากนั้นจึงตรวจวัดผลด้วยเทคนิคเรียลไทม์พีซีอาร์ โดยได้ทำการปรับอุณหภูมิในการทำปฏิกิริยาเพื่อให้มีความจำเพาะในการตรวจวินิจฉัย ผลการทดลองพบว่าเทคนิคมีความจำเพาะสูง ไม่เกิด cross detection และ non-specific amplification จากนั้นทำการทดสอบความไวในการตรวจวินิจฉัยโดยการใช้ positive control ที่เจือจาง 10 เท่าแบบไล่ระดับจาก 10^{8}-10 copies/ µl ผลที่ได้พบว่าเทคนิคนี้มีความไวในการตรวจวัดไมโครอาร์เอ็นเอแต่ละชนิดอยู่ที่ 100 copies/ µl นอกจากนี้ได้ทำการทดสอบสำหรับการนำไปประยุกต์ใช้ทางคลินิกโดยตรวจหาปริมาณไมโครอาร์เอ็นเอจากซีรั่มของผู้ป่วยที่เป็นโรคตับคั่งไขมันจากการศึกษาพบว่าสามารถใช้ตรวจหาปริมาณไมโครอาร์เอ็นเอจากกลุ่มผู้ที่เป็นโรคตับคั่งไขมันได้ นอกจากนี้ผลการทดสอบบ่งชี้ว่าไมโครอาร์เอ็นเอ 29 ซี และไมโครอาร์เอ็นเอ 122 อาจจะเป็นตัวชี้วัดทางชีวภาพที่ดีสำหรับโรคตับคั่งไขมัน โดยสรุปเทคนิคเรียลไทม์พีซีอาร์มีประสิทธิภาพในการตรวจวัดปริมาณของไมโครอาร์เอ็นเอ 29 และ ไมโครอาร์เอ็นเอ 122 ดังนั้นเทคนิคนี้จึงอาจมีประโยชน์ในการตรวจหา และวัดปริมาณไมโครอาร์เอ็นเอ 29 และไมโครอาร์เอ็นเอ 122 เพื่อใช้เป็นตัวชี้วัดทางชีวภาพสำหรับการวินิจฉัยโรคตับคั่งไขมันได้

Key Words: Quantitation, Real-time PCR, MiRNAs
คำสำคัญ: การวัดปริมาณ เรียลไทม์พีซีอาร์ ไมโครอาร์เอ็นเอ

Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs approximately 22 nucleotides (nt) in length that regulate gene expression or translational repression mechanisms. It has been estimated that miRNAs are regulate ～30% of human genes. During miRNA biogenesis, miRNAs are subjected to involve in post-transcriptional regulation, and the elucidation of these mechanisms will improve our understanding of miRNA deregulation in disease progress (Garzon et al., 2009; Kriegel et al., 2012). MiRNAs are normally transcribed by RNA polymerase II or III in the nucleus to generate long primary transcripts, up to several kilobases (primary miRNA) which will be subsequently processed by the enzyme called Drosha and protein called DiGeorge syndrome critical region gene 8 (DGCR8) to become 60-100 nucleotides which is called precursor miRNA (pre-miRNA). The pre-miRNA is exported to the cytoplasm by exportin-5 before it will be cleaved by the Dicer to yield mature miRNA duplex and bound to the RNA-induced silencing complex (RISC). After that, the miRNA will bind to 3′untranslated region (3′UTR) of target mRNA(s). The result of miRNA specific binding can lead to mRNA degradation or translational repression (Garzon et al., 2009; Winter et al., 2009). Levels of miRNAs in serum and plasma samples can be used as perfect biomarkers because they are simple to be approachable in a non-invasive manner (Brase et al., 2010; X. W. Wang et al., 2012). Previous studies found that miR-29 family (a, b and c) and miR-122 have the dominant role to regulate genes in many liver diseases such as liver cancer, hepatitis, metabolic syndrome and fatty liver disease.

The human miR-29 family includes hsa-miR-29a, hsa-miR-29b and hsa-miR-29c. Mature miR-29 family is highly conserved in human, mouse, and rat (Kriegel et al., 2012). The mature miR-29 family members share the same seed sequences (position 2nd – 8th from the 5′ end of miRNA) which determine the

target sequences; it suggested that the sequences on seed region of target genes regulated by miR-29 family are quite similar. MiR-29 family were showed to express aberrantly in multiple cancer types and are involved in complex regulatory process by targeting multiple factors associated with several common pathways, indicating that it can play critical role in apoptosis, metastasis, immune regulation and cell proliferation processes by targeting various genes such as p85 alpha and CDC42, TGF beta, extracellular matrix, INF-grammar and CDK6 respectively (Kriegel et al., 2012; Y. Wang et al., 2013).

Previous study revealed that overexpression of miR-29 family blocks insulin-stimulated glucose uptake via the Akt pathway by inhibiting insulin signaling (He et al., 2007). In 2011, researchers found that miR-29 family are up-regulated via inhibition effects of PECK expression by prevention of the insulin signaling through p85 alpha subunit of PI3K-Akt pathway leading to insulin resistance, which is the most risk factor for the development of non-alcoholic fatty liver disease (NAFLD) and steatosis that links all components from the metabolic syndrome (Pandey et al., 2011). Moreover, from the study of miR-29 in the progression of breast cancer revealed that miR-29 family plays a significant role in mediating loss of N-myc interactor (NMI) and subsequent epithelial-mesenchymal-transition (EMT) (Rostas et al., 2014). Downregulation of ten–eleven translocations 1 (TET1) which is a target of miR-29, influences cancer development such as hepatocellular carcinoma (HCC); hence, miR-29 be employed as prognosis marker and therapeutic target for HCC (Lin et al., 2015). In addition, apoptosis and cell cycle in colorectal cancer (CRC) can be regulated by miR-29b, indicating that this miRNA may be served as a potential prognostic marker of CRC (Inoue et al., 2014).

MiR-122 is an abundant miRNA in the liver accounting for nearly 70% of all miRNAs which is found in mouse, woodchuck and human livers, in human primary hepatocytes, and in cultured liver derived cells, such as mouse Hepa 1-6 cells and human Huh7 cells (Girard et al., 2008). MiR-122 can be detected in the circulation and serum. Moreover, miR-122 plays an important role in liver development, differentiation, homeostasis and functions. Moreover, miR-122 is also a biomarker of liver injury in chronic hepatitis B or C, NAFLD and drug-induced liver damage (Bandiera et al., 2014; Rottiers et al., 2012). Consequently, from the search for miRNA deregulated pathways involved in liver diseases, miR-122, a paradigm for the role of microRNAs in the liver, deserves to be a dominant alternative (Girard et al., 2008).

From the former research, the relationship between serum levels of circulating miRNAs and NAFLD and the correlation between miR-122 and the severity of liver steatosis, these serum miRNAs, especially miR-122 can be considered as a beneficial biomarker for NAFLD (Yamada et al., 2013). The previous study showed that the hepatic and serum miR-122 levels were associated with hepatic steatosis and fibrosis. This serum levels contain inverse correlation between liver fibrosis, and decrease of miR-122 expression associates with advanced fibrosis stage (Miyaaki et al., 2013). As same as in 2011, there may be advantage of miR-122 to identify the patients who have NAFLD in which they developed to significant liver fibrosis (Cermelli et al., 2011). Another study presented that expression of serum miR-122 levels are increased with steatosis in which it causes fibrosis

(Vincent et al., 2014). Moreover, the silence of miR-122 would affect the stimulation of a hepatocarcinogenesis (Takaki et al., 2014). Furthermore, high levels of serum miR-122 are considered to be a satisfying biomarker of prognosis in patients with HCC because it associated with higher overall survival rate in HCC patients (Xu et al., 2015).

The detection of miR-122 and miR-29 can be effective in evaluating the liver injury and fibrosis associated with HBV infection. Since the expression of miR-122 may be associated particularly with the occurrence of liver damage in chronic hepatitis B (CHB) also in liver cancer and low miR-29 expression could correlated to liver fibrosis (Xing et al., 2014). In the present study, we developed techniques for detection and quantitation of miR-29 family and miR-122, this might be useful as a possible biomarker for diagnosis of several diseases.

Objective of the study

To develop the assay based on real-time PCR for detection and quantitation of miR-29 family and miR-122 which might be useful as possible biomarkers for diagnosis of NAFLD.

Methodology

Preparation of positive control miRNAs

Nucleotide sequences of miR-29 (a, b and c) and miR-122 were designed by using data obtained from miRBase (http://www.mirbase.org). RNA positive controls for each miRNA were generated by *in vitro* transcription. DNA templates for *in vitro* transcription composed of short and long oligonucleotides. The short oligonucleotide was synthesized based on T7 promoter sequence whereas the long oligonucleotide was synthesized based on the complementary sequence (antisense) of the T7 promoter linked to the 5′-end of each miRNA. The sequences were described in Table 1. After that, duplex DNA templates were prepared (Figure 1) by mixing 10 µM of each oligonucleotide in a total volume of 10 µl then incubate at 90°C for 1 minute and followed by 37°C for 1 hour. *In vitro* transcription was performed by using Ribomax large scale RNA production system T7 (Promega) according to the manufacturer's instruction. Briefly, the reaction mixture (20 µl) included 4.0 µl of 5X T7 transcription buffer, 6.0 µl of 25mM rNTPs mix (ATP, CTP, GTP, UTP), 5.0 µl of DNA template (5 µg), 40 units of T7 RNA polymerase and 3 µl of nuclease free water. After that the transcription reaction was occurred by incubating at 37°C for 4 hours then followed by adding 5 µl of RQ1 RNase free DNase and incubate at 37°C for 15 minutes. The *in vitro* transcribed miRNAs were extracted by microRNA purification kit (Norgen) following the instruction protocol from the company.

The concentration of each miRNA was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific) and then calculated in terms of copies/ µl. These miRNAs were then used as a positive control and standard miRNA for detection and quantitation of miRNA, respectively. The miRNAs were extracted from

Table 1 Primer and oligonucleotides used in this study.

Primers / oligos	Sequence (5′→ 3′)	Strand	Application
T7 (short)	TAATACGACTCACTATAGGG	Sense	Template for *in vitro* transcription
T7+ miR-29a (long)	CCGATTTCAGATGGTGCTACCCTATAGTGAGTCGTATTA	Antisense	
T7+ miR-29b (long)	CCTGATTTCAAATGGTGCTACCCTATAGTGAGTCGTATTA	Antisense	
T7+ miR-29c (long)	ACCGATTTCAAATGGTGCTACCCTATAGTGAGTCGTATTA	Antisense	
T7+ miR-122 (long)	ACACCATTGTCACACTCCACCCTATAGTGAGTCGTATTA	Antisense	
Stem loop (SL) poly (A) (Mei et al., 2012)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAAAAAAAAAAAAAAAAAVN	Sense	Reverse transcription
miR-29a_F	TAGCACCATCTGAAATCGG	Sense	Real-time PCR
miR-29b_F	TAGCACCATTTGAAATCAGG	Sense	
miR-29c_F	TAGCACCATTTGAAATCGGT	Sense	
miR-122_F	TGGAGTGTGACAATGGTGT	Sense	
Universal qPCR_R (Mei et al., 2012)	GCAGGGTCCGAGGTATTC	Antisense	

Note: Underline indicates T7 promoter sequence. F refers to forward primer and R refers to reverse primer.

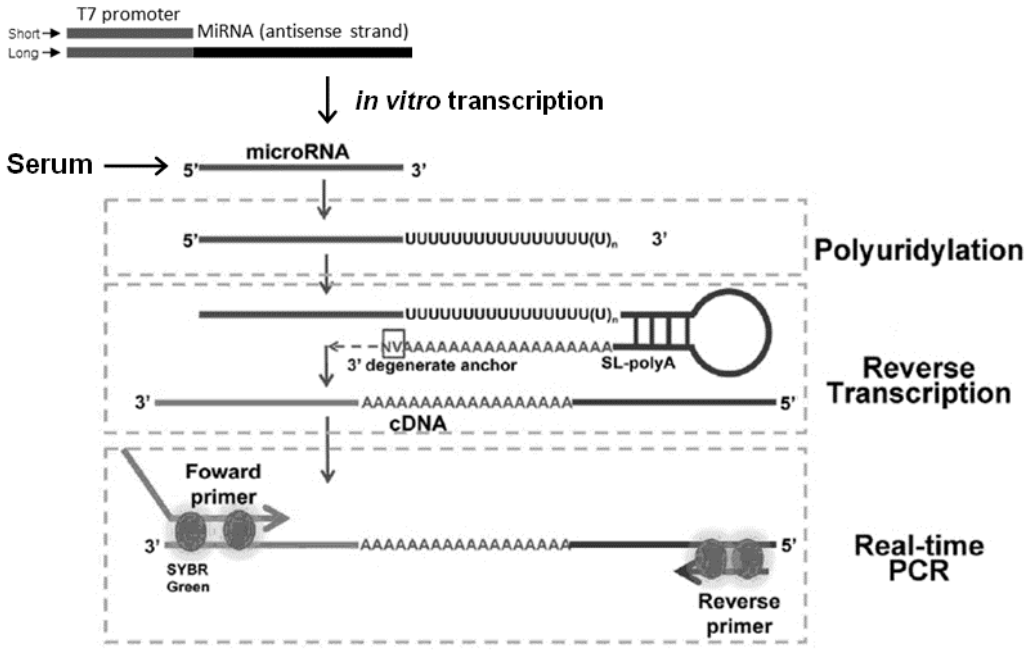

Figure 1 Schematic description of assay design for miRNA quantitation (Modified from Mei et al., 2012).

serum or generated from the *in vitro* transcription. Mature miRNAs were polyuridylated by poly (U) polymerase and then reverse transcribed by using stem loop (SL) poly A primer and reverse transcriptase. Real-time PCR based on SYBR green dye was performed by using miRNA specific forward primer and universal reverse primer.

Serum samples from NAFLD patients

Totally, serum samples (N=63) were collected from NAFLD patients who attended health examinations in King Chulalongkorn Memorial Hospital, Bangkok,

Thailand. All participants provided written informed consent. The study protocol was approved by Institutional Review Board (IRB No. 357/57), Faculty of Medicine, Chulalongkorn University. Serum miRNAs were extracted from 100 µl of serum by using microRNA purification kit (Norgen) according to the manufacturer's instruction.

Reverse transcription

The technique for quantitation of miRNA used in this study was described previously (Mei et al., 2012). The principle of the assay was illustrated in Figure 1. Briefly, mature miRNAs were polyuridylated by poly (U) polymerase and then reverse transcribed by using stem loop (SL) poly A primer and reverse transcriptase. After that real-time PCR based on SYBR green dye was performed by using miRNA specific forward primer and universal reverse primer. Polyuridylation reaction included 2.5 µl of 10x NE buffer, 0.25 µl of 50 mM UTP, 40 units of RNase inhibitor, 2 units of poly (U) polymerase (New England BioLabs Inc.), 100 pmol of miRNA and nuclease free water in a final volume of 25 µl then incubate at 37°C for 10 minutes. After that reverse transcription was performed by using 12.3 µl of polyuridylated miRNA, 4.0 µl of 5x RT reaction buffer, 0.2 µl of 10 µM stem loop (SL) poly A primer, 2 µl of 10 mM dNTPs mix, 20 units of RNase inhibitor, 200 units of RevertAid reverse transcriptase (Thermo scientific) and nuclease free water in a final volume of 20 µl.

Quantitative real-time PCR

Quantitation of miRNA was carried out in Step One Plus real-time PCR (Applied Biosystems, Foster City, CA) based on SYBR dye. Real-time PCR reaction mixture composed of 6.25 µl of 2x Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific), 0.4 µl of 10 µM of each primer, 1 µl of cDNA and nuclease free water in a final volume of 12.5 µl. The sequence of primers used in this study was summarized in Table 1. Thermal profiles were optimized for each miRNA as the following: initial denaturation at 95°C for 3 minutes and then 50 cycles of amplification including 95°C for 15 seconds and 60°C (miR-122) or 62°C (miR-29 family) for 30 seconds.

Construction of standard curve

For each miRNA, serial 10-fold dilutions of the standard *in vitro* transcribed miRNAs ranging from 10^8 to 10 copies/ µl were detected by real-time PCR assay and used to prepare the standard curve for quantitation of miRNAs from patient specimens. Standard curve was constructed by plotting the Ct value against the amount each serially diluted standard miRNA. Then the standard curve also was used for determination of amplification efficiency and detection limit of the assay.

Results

Detection of miR-29 family and miR-122 based on real-time PCR

In this study, the assays based on real-time PCR for detection and quantitation of miR-29 family (a, b & c) and miR-122 were successfully developed. The designed primers and optimized conditions yielded effective and specific amplification of the miRNAs without any non-specific amplification or primer dimers (negative control in Figure 2). In addition, the specificity of the assay was evaluated by using miRNA specific primers to test against the *in vitro* transcribed miRNA positive control (10^6 copies / µl). Results revealed that miRNA specific primers provided impressive specificity under the optimized thermal profile without any cross-amplification with other miRNAs (Table 2). Moreover,

the assay was also validated in terms of capability for detecting miRNA from clinical samples. Serum miRNAs were extracted from NAFLD patients (N=63) then followed by polyuridylation, reverse transcription and real-time PCR detection. Result shown that the assay can be used to detect miR-29 family and miR-122 extracted from serum of NAFLD patients (Figure 2). However, the capability of detection was differently observed for each miRNA. Results revealed that miR-29a was detected in 52/63 samples (82.5%) whereas miR-29b was detected only in 22/63 samples (34.9%). Interestingly, miR-29c and miR-122 were frequently detected in 61/63 samples (96.8%) and 60/63 samples (95.2%), respectively (Table 2).

Performances of the real-time PCR assay

For each miRNA, standard curve was constructed by using 10-fold serial dilutions of the standard *in vitro* transcribed miRNAs ranging from 10^8 to 10 copies/ µl. Then standard curves were used for analysis of the overall performance of the real-time PCR assay in terms of detection limit and amplification efficiency. Results revealed that the limit of detection for miR-29a, miR-29c and miR-122 was approximately 100 copies/ µl whereas those found in miR-29b was around 1000 copies/ µl (Figure 3). According to the standard curves, amplification efficiency of miR-29a was about 97.08% (slope = -3.394 and R^2= 0.994) whereas those found in miR-29b was approximately 88.35% (slope = -3.637 and R^2= 0.996). Similarly, amplification efficiency of miR-29c was around 89.54% (slope = -3.601 and R^2= 0.996) while those observed in miR-122 was approximately 101.06% (slope = -3.297 and R^2= 0.999).

Absolute quantitation of miR-29 and miR-122 from serum of NAFLD patients

The expression levels of miR-29 family and miR-122 in serum of NAFLD patients were determined via absolute quantitation by comparing the Ct value of each sample with the standard curves. Results revealed that the median of serum miRNA level of the miR-29a, miR-29b, miR-29c and miR-122 were approximately 184.9, 2.4, 159.4 and 32161.0 copies/ µl, respectively (Figure 4). The level of miR-29b was significantly lower than other miRNAs. The miR-29a and miR-29c provided similar expression pattern which most of the samples yielded expression level ranging from 10-10^3 copies/ µl while minority of sample (N=7) yielded very high expression levels around 10^5 copies/ µl. On the other hands, the levels of miR-122 in most samples were ranged from 10^2-10^4 copies/ µl.

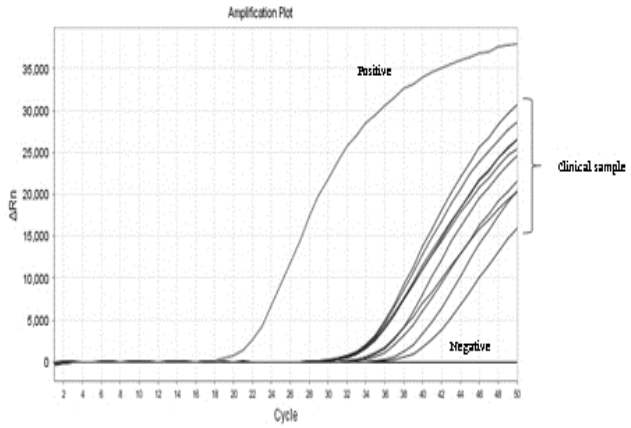

Figure 2 Representative results for the validation of the assay in terms of detecting miRNA extracted from serum sample of NAFLD patients. Positive refers to the positive control of miRNA (10^6 copies / µl) whereas negative refers to no template control.

Discussion and Conclusion

Quantitative real-time PCR is one of potential techniques for investigation of gene expression in terms of quantitative and qualitative manner. This technique has been used in life sciences and medicine because of its high sensitivity, specificity and accuracy. Moreover, it is widely adapted to quantify miRNAs in general

research (Mei et al., 2012). In this study, the assay based on real-time PCR was developed for detection and quantitation of miR-29 family and miR-122. The result of real-time PCR revealed the limit of detection for miR-

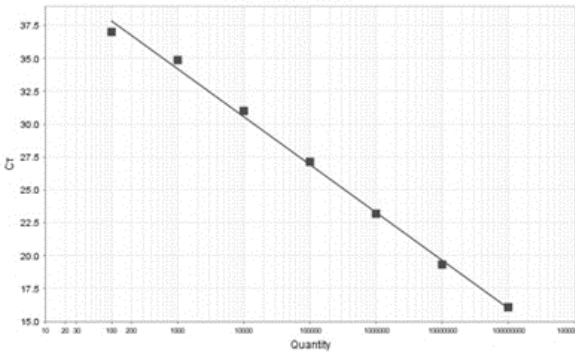

Figure 3 A representative standard curve constructed from serial 10-fold dilutions of the *in vitro* transcribed miRNAs ranging from 10^8 to 10 copies/ µl.

Table 2 Validation of the assay in terms of specificity and capability of miRNA detection from serum.

Assay	Specificity test*				Detection of miRNA from serum sample	
	miR-29a	miR-29b	miR-29c	miR-122	No. of detected	% of detected
miR-29a	+	-	-	-	52 / 63	82.5
miR-29b	-	+	-	-	22 / 63	34.9
miR-29c	-	-	+	-	61 / 63	96.8
miR-122	-	-	-	+	60 / 63	95.2

* Positive controls (10^6 copies / µl) were used for the specificity test; (+) = positive detection; (-) = no cross amplification

29a, miR-29c and miR-122 were approximately 100 copies/ µl, indicating that in order to detect those miRNAs, one microliter of sample needs to contain at least 100 copies of the miRNA. On the contrary, in a case that there are less than 100 copies of miRNA, it yieldes undetectable results. The limit of detection for miR-29b was around 1000 copies/ µl, indicating lower efficiency of the primers for detection. As a result, the miR-29b was detected only in 22/63 serum samples of NAFLD patients (34.9%) which relatively lower than those observed in miR-29a (82.5%), miR-29c (96.8%) and miR-122 (95.2%). However, the acceptable limit of detection for real-time PCR assay ranged from 10-10^3 copies/ µl (Payungporn et al., 2008). Basically, the standard curve obtained from real-time PCR should yield efficiency of amplification (90 to 110%), slope (-3.1 to -3.6) and R^2 value (0.985 to 1.000) (Payungporn et al., 2008). The standard curves of miRNAs obtained from this study were in agreement with the criteria described above, indicating that the overall performances of the real-time PCR assay were acceptable.

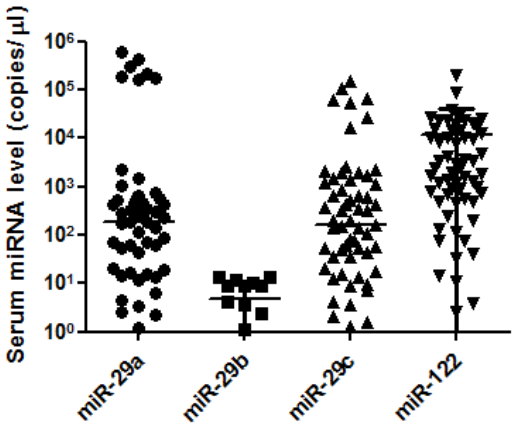

Figure 4 Scatter plots of differentially expressed miRNAs in serum of NAFLD patients. Serum miRNA expressions were obtained from real-time PCR analysis based on absolute quantitation. Each emblem represents a data derived from each sample.

Furthermore, serum miR-29 family and miR-122 from NAFLD patients were also detected and quantitated by real-time PCR assay. The results suggested that the assay may be used to detect and quantify miR-29 family and miR-122 extracted from serum of NAFLD patients. From the scatter plots, serum level of miR-122 and miR-29c may useful in the identification of NAFLD patients. From 63 samples, the miR-122 was detected up to 60 which accounted for 95.2% and miR-29c was detected up to 61 which accounted for 96.8%. From this data, miR-122 and miR-29c have the ability to detect more than 95%, implying that these two miRNA may be served as potential, efficient and interesting biomarkers in the future. Previous study revealed that the serum levels of miR-122 in NAFLD patients are up-regulated compared to the control group ($P < 0.001$) (Yamada et al., 2013). The comparison between healthy controls and NAFLD patients stated that miR-122 levels were increased by 7.2-fold ($P < 0.0001$) (Cermelli et al., 2011). However, there is less research described the relationship between miR-29 family and NAFLD. Most of the research study linked miR-29 family through metabolic pathways (He et al., 2007; Pandey et al., 2011). Therefore, further investigation on miR-29 should be performed in order to compare the levels between normal controls and patients with different stage of NAFLD disease.

In conclusion, the real-time PCR assay described in this study may be used to detect and quantitate miR-29 family and miR-122. The development of this technique is another essential way which can continue to support the research on miR-29 family and miR-122. The assay might be useful and attractive for detection and quantitation of miR-29 family and miR-122 as possible biomarkers for NAFLD in the future.

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