DESIGNING A STEM-LOOP RT-qPCR METHOD FOR MEASURING miRNA-16 EXPRESSION IN HUMAN PLASMA SAMPLES

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SUMMARY

MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNA molecules. They have the potential use as excellent biomarkers in many disease states. Stem-loop RT-qPCR is known as a high specific and consistent technique for measuring miRNA levels. In this study, a stem-loop RT-qPCR is designed and optimized for circulating miRNA quantification in human plasma. Total RNA was extracted from plasma following by the reverse transcription (RT) to cDNA using gene-specific primer. Subsequently, the expression levels of miRNA-16 were called out by the amplification curve – the fluorescent signal released during real-time PCR. The sensitivity, stability and specificity of the assay was consecutively estimated by the ability to amplify the concentration of template, the consistence of amplification and the melting peak. The primary results revealed that 85.7% of samples were amplified during PCR whereas only one did not appear in the amplification plot. Moreover, there is not significant difference ($p > 0.05$) of expression levels among samples, which indicates the stability of this PCR method. With small numbers of samples tested and non-specific products appearance, the specificity of the assay was low (16.7%). Up to date, a stem-loop RT-qPCR primer has been designed for miRNA-16 expression analysis. However, the optimal method could be obtained after being validated with stability and higher specificity.

Keywords: Circulating miRNA, miRNA, miRNA-16, plasma, Stem-loop RT-qPCR.

INTRODUCTION

Accumulating evidence in the past several years has highlighted the potential use of microRNA (miRNA) as excellent biomarkers in many disease states, including cancer. These miRNAs support surgeons in both guiding the inital diagnosis of cancer and monitoring after cancer resection (Rice *et al*., 2015). In clinical studies, certain miRNAs were used as biomarkers for diagnosis of lung, breast, colon, and esophageal cancers (Tripathi *et al*., 2019; Yao *et al*., 2019; Li *et al*., 2019; Sabry *et al*., 2019). Researchers also showed that approximately 50% of miRNAs are post-transcriptional regulators and few of them can play both the tumor suppressive and the oncogenic role (Slack, Weidhaas, 2008). Specifically, it is determined that some miRNAs are dominantly expressed in specific tissues and reduced expression levels when the tissues are in tumor stage.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs molecules with a length of 18 - 25 nucleotides derived from ~70-nt precursors. They can directly bind to 3'-untranslated region (3′-UTR) of target mRNAs, causing transcript destabilization, translational repression or both (Hamam *et al*., 2017), thus these molecules play critical roles in numerous regulatory pathways like cell proliferation, signal transduction, apoptosis, and metastasis (Bartel, 2004). miRNAs which are present in the circulatory systems of patients are called circulating miRNAs. They are different from cellular miRNA in functioning inside cells. These miRNAs are eitheir bound to ribonucleoprotein complexes or released from cells in lipid vesicles, exosomes or apoptotic bodies. They have high resistance to RNase activity, long-term storage and multiple freeze-thaw cycles, which are advantageous characteristics of biomarkers. With the great increase in reported studies of miRNA, there has been little standardization of methodology, making it difficult to compare different studies with each other. Accurate quantification of miRNAs in plasma poses a number of challenges because of their low abundance and small size. Quantitative reverse transcriptase real-time (qRT-)PCR is a widely used and highly sensitive method that requires only small amounts of input RNA. Standard and quantitative PCR methods require a template that is at least twice the length of either of the specific forward or reverse primers, each typically ~ 20nt in length. Thus, the target minimum length is ≥ 40 nt, making miRNAs too short for the standard RT-qPCR method (Kramer, 2011). Among all types of quantitative PCR, stem-loop RT-qPCR is a cost- effective, convenient and consistent technique for measuring individual miRNA in tissue or cultured cells. The technique is highly specific for miRNAs, but not for genomic DNA. Particularly, the method is specific to mature miRNAs but not to their precursors, even if both are present in the same concentration. Another point is that the total RNA concentration at the beginning can be as low as 20 picograms to quantify miRNAs (Chen, 2005). Stem-loop RT-qPCR requires

preparation of separate cDNA templates for each analyzed miRNA as reverse transcription occurs in the presence of a miRNA-specific stem-loop reverse primer. In quantitative analysis, cDNA templates are amplified and detected by fluorescent signal. With the expanding field of miRNA research and the promise of certain miRNAs as biomarker, it is desirable that methods of detection need to be consistent between investigators (Rice *et al*., 2015). Moreover, stem-loop RT-qPCR is rarely applied in cancer diagnosis and miRNA functional studies in Vietnam. The result of this study may suggest another sensitive and specific miRNA-diagnostic method for Vietnamese scientists and researchers.

In studies of plasma miRNAs, microRNA (miR)-16 is expressed in all samples, consistently present and sufficiently abundant in plasma. In 2015, Rice reported that miR-16 was present in both control and patient groups, also its expression level in plasma was higher than other miRNAs studied. The stable concentration of these miRNAs in plasma were evaluated (Benson, Skaar, 2013), and the results show that the presence of miR-16 in plasma was stable up to 12-hour incubation at room temperature. Therefore, miR-16 was chosen as the target material for this project. In general, this study aims to design and optimize stem-loop RT-qPCR method for miRNA-16 quantification and apply that optimal protocol further in miRNA investigations.

MATERIALS AND METHOD

Sample preparation

Collecting human plasma samples and total RNA extraction

Seven whole blood samples were collected then stored in 2mL tubes containing EDTA. Plasma samples were collected then centrifuged for 10 minutes at 16,000 xg and 4^{o}C. The cleared supernatant was carefully transfered to a new tube without disturbing the pellet. Samples were maintained at 2 - 8°C until further processing, if not 0.5 ml aliquots were kept frozen at -80°C for longer storage. The detailed protocol follows the instructions from miREasy QIAGEN kit.

A commercial kit with some modification was used to extract total RNA from plasma samples.The whole process for RNA extraction follows Life Technology - manufacturer's instructions:

After RNA extraction, Nano - drop Spectrophotometer was used to check RNA quality and quantity at wavelength 260 and 280 nm. All RNA samples, which were used in the study, were considered as high purity whose A260/A280 values fell between 1.8 and 2.1.

Stem-loop RT-qPCR design

Three primers which are designed to transcribe reversely the mature microRNA isolated to cDNA and amplify it to various amplicons and prevent the occurrence of other variations in the primer region. The true target sequence of the mature miRNA was obtained from miRBase website (http://www.mirbase.org/). The web Fluiogenico.cl is used for Design Stem-loop RT-qPCR primers. Primer Blast and Oligo Analyzer were used for checking the specificity and the melting temperature of the chosen primers. Multiple primer analyzer (Thermofisher) is used for checking the secondary structure of the chosen primers. Other manually designing also applied:

- **Stem-loop primer** combines 44 nt of the stem-loop sequence of Chen *et al*. 2005, 5′ - GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GATACG AC -3′ with the complement of the six 3′ nt of the mature miRNA sequence.
- **Forward qPCR primer**. Take the first sixteen 5′ nt from the mature miRNA sequence, then add six nucleotides to the 5′ end.
- **Reverse qPCR primer**. By using the same 44 nt stem-loop sequence for all RT primers, a universal primer can be derived from sequences within the stem-loop. The recommended primer is 5′ -GTG CAG GGT CCG AGG T-3′

Moreover, there were some modifications of forward primer recommended from (Kramer, 2011) since it was confirmed that this change will increase stable primer binding and PCR quality.

Stem-loop RT-qPCR method optimization

Reverse Transcription

RT procedure was set as recommend of Agilent Technologies kit. RNA and primer denaturation takes place within 5 minutes at 65^{o}C following by the annealing step at gradient temperature in 5 minutes. Subsequently, a 30-minute incubation for first-strand synthesis reaction at 42^{o}C is effective for activity of Reverse Trancriptase. After all, samples were kept at 4^{o}C until further processing.

Real-time PCR

cDNA samples will be used for further optimization to find out the best real-time PCR procedure. The Annealing temperature (Ta) = 60ºC. PCR thermal cycle was set as recommend of Bioline kit. The first step is polymerase activation, which takes place within 2 minutes at 95ºC. Subsequently, DNA was denatured for 5 seconds at 95ºC following by the annealing step at 60ºC in 10 seconds. After all, extension step is set at 72ºC in 10 minutes. PCR cycles were repeated 40 times.

RESULTS AND DISCUSSION

Sample preparation

7 case samples were extracted successfully by using isolation kit. These samples obtained the concentration above 10 ng/uL and high purity of total RNA (the A260:A280 ratio in the range of 1.8 - 2.2) which meant that the total RNA were highly purified and quantified for PCR analysis.

Primer design

Using several websites and manually modifying the stem-loop RT-qPCR primer set has been designed with their sequence and characteristics seen in Table 1.

Table 1. Sequence and characteristics of the stem-loop RT-qPCR primers set

Primer	Sequence (5'-3')	Tm (ºC)	Length (bp)	Human RNA alignment
Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACCGCCAA	20 (6-annealing nucleotides)	50	Not found any identity
Forward	CACGTATAGCAGCACGTAAATA	56.46	22	100% identity
Reverse	CCAGTGCAGGGCCGAGGTA	58.01	16	No alignment

Stem-loop RT-qPCR optimization and evaluation

PCR was run as table 2 and RT reactions were set up as table 3. Three times running HRM reactions for 7 samples and negative control. Due to different concentration of RNA after extraction, we diluted samples and used 10-20 ng of RNA per reaction so as to measure expression levels of miRNA-16 (Table 2).

Table 2. Modified RT protocol

Steps/ Temperature - Duration	Reagents	
	Components	**Volume (uL/ rxn)**
Denaturation/ 65ºC – 5min Annealing/ 16ºC – 5min	AccuScript RT buffer	1.0
	Stem-loop primer	0.6
	dNTP mix	1.0
	RNase-free water	
	RNA (10-20ng)	**Variable**
	Total	4
First-strand cDNA synthesis/ 42ºC – 30min	DTT	0.5
	AccuScript Reverse Transcriptase	0.5
Cooling/ 4ºC		

Table 3. Modified PCR protocol

Reagents	
Components	**Volume (uL/ rxn)**
SensiFast HRM mix	5
Forward primer	0.4
Reversed primer	0.4
cDNA	2.0
Nuclease-free water	2.2
Thermal cycle of PCR in HRM analysis	
Pre-incubation	95°C – 2min
Amplification (40 cycles)	95°C - 5 s **60°C - 10 s** 72°C - 10 s
High Resolution Melting	95 °C - 60 s 40°C - 60 s 65°C - 1 s 97°C - 1 s

The method used was evaluated by three parameters: sensitivity, and stability, specificity.

Sensitivity. The ability of the method showing the amplification curve. The results reached to over 85% of sensitivity in which 6/7 samples (figure 1) were amplified efficiently and their curves are higher than that of negative control during PCR.

Stability. Evaluate the ratio between expression level (Cq) and amount of RNA per reaction. Despite limitation exists in the study such as limited experimented time and long waiting time for chemicals, this method is quite stable. Expression level of miRNA-16 was found in the literature to be consistent on healthy group, therefore it is probable that similar RNA concentration leads to similar levels of expression. T-test was used for evaluating the difference in expression levels among samples, the test results were described in Table 4. With p-value > 0.05, there is not significant difference in expression levels among samples, proving our expectation.

Table 4. Using t-test for stability

Sample	Amount of RNA per reaction (ng)	Expression level (Cq)	Cq/amount of RNA
1	12.4	27.5	2.2
2	10.0	38.5	3.9
3	12.9	28.5	2.2
5	15.2	34.5	2.3
6	14.4	31.3	2.2
7	18.3	26.5	1.5
P value (t-test)		**0.24**	

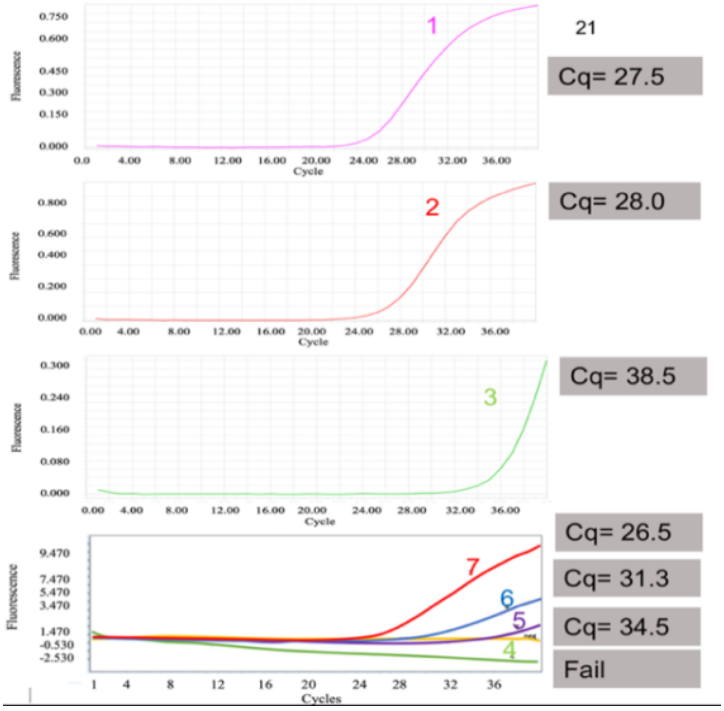

Figure 1. Amplification plot of each sample

Specificity. Evaluate the method's specificity through melting peak analysis. Basing on the result of primer design, the estimated peak for miRNA-16 was 71.8ºC. We luckily have the very specific result on the first run. However, there were mistakes during perfomance leading to appearance of non-specific products on the other runs. It is found in the literature that observed peak would be 2 - 3ºC difference compared to the estimated one. From these reasons, we can confirm one of the peak is our target miRNA. Ingeneral, the specificity of the assay was only 16.7%. Figure 2a, b, c, d, e and f illustrated the melting peak result of all samples.

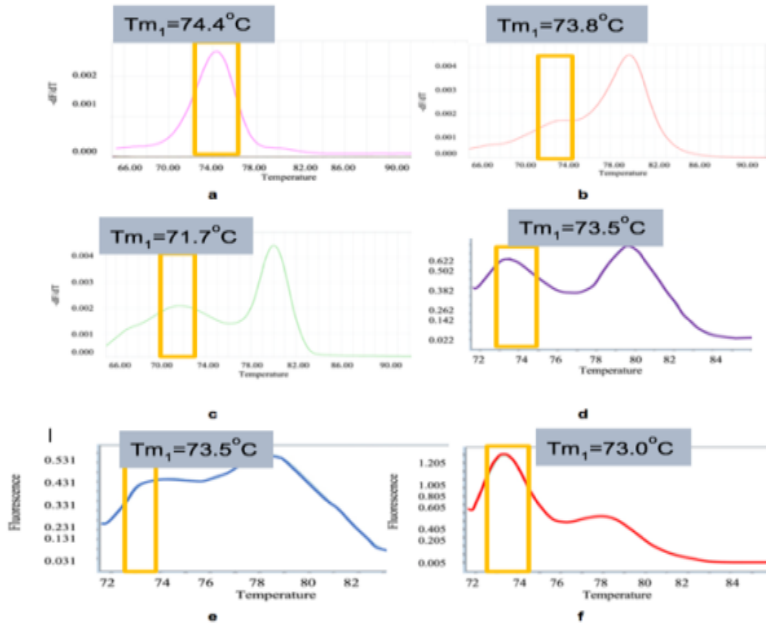

Figure 2. The melting peak analysis

In this study, stem-loop RT-qPCR has been designed for miRNA-16 quantification. This method is potential for miRNA quantitative analysis because of its high sensitivity, stability and appropriate for applying in small-scale laboratory. However, limitations in this study exist. There was no validation method for the reverse transcription step. Although we kindly accepted the concentration of total RNA as the concentration of miRNA, the PCR results conducted non-specific products. It can be explained that there may be primer-dimer structure, or the annealing temperature is not optimal for PCR.

CONCLUSION

With the expanding field of miRNA research and the promise of certain miRNAs as biomarker, it is desirable that methods of detection need to be consistent between investigators. Moreover, stem-loop RT-qPCR is rarely applied in cancer diagnosis and miRNA functional studies in Vietnam. Up to date, a stem-loop RT-qPCR has been designed for miRNA-16 expression analysis. However, the optimal method could be obtained after being validated with bigger size of sample and higher specificity.

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XÂY DỰNG PHƯƠNG PHÁP STEM-LOOP RT-qPCR ĐỊNH LƯỢNG miRNA-16 TRONG MẪU HUYẾT TƯƠNG

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TÓM TẮT

MicroRNA (miRNA), một họ các phân tử RNA nội bào không mã hóa (non-coding RNAs) đóng vai trò điều hòa sau phiên mã biểu hiện gen, từ đó ảnh hưởng những quá trình cốt yếu trong hệ thống sinh học, bao gồm phát triển ung thư. Các phân tử này có khuynh hướng được sử dụng như dấu chứng sinh học trong các giai đoạn phát triển bệnh. Stem-loop RT-qPCR được biết như một phương pháp đặc hiệu cao và ổn định để định lượng biểu hiện miRNA ở tế bào mô. Trong nghiên cứu này, phương pháp stem-loop RT-qPCR được thiết kế và tối ưu để định lượng miRNA tuần hoàn ở huyết tương người, loại mô đã được kiểm chứng là có nồng độ miRNA thấp hơn so với các tế bào mô khác. Lượng RNA tổng được tách chiết từ huyết tương và phiên mã ngược thành DNA bổ sung (cDNA) bằng mồi đặc hiệu. Sau đó, biểu hiện của miRNA-16 được khuếch đại và ghi nhận bởi các tín hiệu huỳnh quang trong real-time PCR. Độ nhạy, độ ổn định và độ đặc hiệu của phương pháp lần lượt được đo lường bởi khả năng khuếch đại nồng độ khuôn mẫu, sự thống nhất trong quá trình khuếch đại và kết quả đỉnh nóng chảy. Các kết quả chính thu nhận 85,7% mẫu được khuếch đại sau PCR trong khi chỉ có một mẫu không xuất hiện sự khuếch đại. Hơn nữa, không có sự khác biệt lớn về sự biểu hiện giữa các mẫu, chứng minh độ ổn định của phương pháp PCR này. Với số lượng mẫu nhỏ và sự xuất hiện của sản phẩm phụ, độ đặc hiệu khá thấp (16,7%). Nghiên cứu này đã thiết kế thành công mồi stem-loop RT-qPCR cho các phân tích biểu hiện miRNA-16, tuy nhiên phương pháp cần được tối ưu thêm để gia tăng độ đặc hiệu.

Từ khóa: miRNA tuần hoàn, miRNA, miRNA-16, huyết tương, Stem-loop RT-qPCR.

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