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Highly specific real-time quantification of diverse microRNAs in human samples using universal primer set frame

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ABSTRACT

In this study, one group of universal primer set frame, composed by one reverse transcription (RT) primer frame and a pair of quantitative real-time polymerase chain reaction (qRT-PCR) primer frames, was elaborately screened and designed by homebuilt software for sensitive and specific quantification of diverse miRNAs. The universal primer set frame can be applied for multiplex miRNAs detection by simply changing the RT-X part of RT primer frame and RP-Y part of qRT-PCR reverse primer frame based on target sequence. The maximum similarity of RT-Y, RT-Z and qRT-PCR forward primer to the human genome and human transcriptome is less than 76%, ensuring the high specificity in human sample detection. The high sensitivity and broad dynamic linear range of the developed approaches by using designed primer set frame were demonstrated on the *in vitro* detection of miR-21 and miR-155, with dynamic range of 10 fM to 10 nM and detection limit of 3.74×10^{-15} M and 5.81×10^{-15} M for miR-21 and miR-155, respectively. In particular, the developed assays also have high sequence specificity which could clearly discriminate a single base difference in miRNA sequence. The contents of miR-21 and miR-155 in tissue and serum samples have been successfully detected using the developed assays. Results indicated that miR-21 and miR-155 were elevated in cancer tissue and serum specimens than that of normal samples, implying the developed assays hold a great promise for further application in biomedical research and early clinical diagnosis. More importantly, the primer set frame can be universally used in any miRNA investigation.

Introduction

MicroRNAs (miRNAs), first discovered in *Caenorhabditis Elegans* in 1993, are short non-coding RNAs (19–25 nucleotides (nt)) that play important roles in various physiological processes through the post-transcriptional regulation of gene expression [1–4]. Aberrant expression of miRNAs is involved in many diseases, such as cancer, diabetes, cardiovascular disease, Alzheimer's disease and immune disease, and so on [5–9]. Recently, miRNAs have been considered as a new class of biomarkers for the diagnosis of cancers and other diseases [5,10–12]. However, miRNA detection is challenged by the characteristics of miRNA, including small size, sequence homology among family members, low abundance in total RNA samples, different melting temperatures and susceptibility to degradation [13]. Therefore, universal, sensitive and specific strategies for detecting miRNAs are imperative, not only for better understanding of the biological functions of miRNAs, but also for clinical diagnosis.

To improve the detection sensitivity, flexibility, and adaptability, various new strategies have been developed for quantification of miRNAs, such as colorimetric-based assay [14], fluorescence-based assay [13,15–19], bioluminescence-based assay [20], electrochemical-based assay [21,22], surface enhanced Raman scattering-based assay [23] and miRNA-Seq-based assay [24,25]. Colorimetric-based detection method is attractive because the setups are relatively simple. However, colorimetric method is limited in quantification of miRNA due to its lower sensitivity [26]. For the fluorescence-based probe assay, each miRNA requires a specific probe which increases the experimental cost in multiplex miRNAs detection [15]. The bioluminescence-based assay makes use of the bioluminescent protein of *Renilla luciferase* (Rluc) as the label and is very simple and rapid, but its drawback is signal-off instead of signal-on [20]. Various electrochemical miRNA biosensors have been fabricated. However, electrochemical detection needs long detection time (> 4 h) [21,27,28]. Surface enhanced Raman scattering (SERS), known as a promising ultrasensitive technique even capable of detecting a single molecule, has long been recognized as a powerful tool for trace biomarker analysis. However, due to the high similarity among miRNA family members, different miRNAs have similar SERS spectral signatures, and overlapping Raman bands [23]. The miRNA-

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Seq, a high-throughput miRNAs analysis approach, is the only platform capable of discovering new miRNAs. While, disadvantages of RNA-Seq are the high cost per sample and the complexity of the workflow and data analysis. Also, the precision of quantitation is poor for the low abundant miRNAs [25,29]. Besides those methods, northern blotting, microarray, reverse transcription polymerase chain reaction (RT-PCR) and next generation sequencing (NGS) are four major approaches to determine levels of miRNAs expression at present [27]. Northern blotting and microarray are the classical tools for miRNA detection. However, the sensitivity and specificity of these methods do not satisfy the detection of the low abundant miRNAs [30–32]. NGS might be used for rapid evaluation of absolute miRNA levels, but it is usually costly and less accurate due to the introduction of some errors in several steps [27]. The RT-PCR is the most practical method for miRNA detection, even though it needs precise control of temperature cycling. However, the use of locked nucleic acids (LNA), stem–loop probes, molecular beacons, or ribonucleotide modified DNA probes make RT-PCR costly and complex (indicate probe design) [33,34]. The TaqMan based miRNA assay, one practical method for miRNA detection, uses hydrolytic probes that are costly to produce and do not allow discriminating the specificity of the assay by melting curve analysis [29]. Therefore, strategies for cost-effective, sensitive and specific detection of miRNAs using PCR as a tool are in urgent need.

To date, more and more new miRNAs have been certified as biomarkers of diseases [10,11,35–37]. Among them, miR-21 and miR-155 are two oncogenic miRNAs that modulate the expression of multiple cancer-related target genes and have been shown to be overexpressed in various human tumors [12,20,36–38]. Therefore, miR-21 and miR-155 were used as models to evaluate the feasibility of the screened universal primer set frame and the developed assays. Two developed sensitive assays also have high sequence specificity to discriminate the perfectly complementary target and the mismatched strands. Therefore, the universal, sensitive and specific assays by using universal primer set frame have potential to become promising miRNAs quantification methods in biomedical research and clinical diagnosis.

Materials and methods

Reagents, materials and instruments

The HPLC-purified DNA and HPLC-purified RNA were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the oligonucleotides were listed in Table 1 and Table S1. RNase inhibitor, dNTP and DEPC-treated water were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China; DEPC, diethylpyrocarbonate). AceQ qPCR SYBR Green master mix and M-MLV (H-) reverse transcriptase were purchased from Vazyme Biotech Co., Ltd (Nanjing, China). Trizol® reagent was purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (USA). Penicillin and streptomycin were purchased from Beyotime Biotechnology (Shanghai, China). The real-time fluorescence measurement was performed with a LightCycler® 96 Real-Time PCR System (Roche Diagnostics, Germany). An OD-1000+ UV-Vis spectrophotometer (One Drop® Technologies, China) was used for absolute quantification of DNA and RNA. All chemicals and solvents were of analytical grade purity.

Cell lines used as follows: human breast cancer cells (MDA-MB-231, MCF-7), human epithelioid cervix carcinoma cell (Hela) and human normal liver cell (HUVEC) were all purchased from American Type Culture Collection (ATCC, Manassas, VA,USA).

Cell culture and total RNA extraction

The cell lines (MDA-MB-231, MCF-7, Hela and HUVEC) were cultured in DMEM supplemented with 10% FBS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) in 5% CO_2 at 37 °C. The total RNA in cell lines, tissue specimens and serum specimens were extracted by Trizol® reagent following the instructions of the manufacturer.

Table 1
Sequences of the oligonucleotides in this work.

Name	Sequence（5'→3'）
miR-21	UAG CUU AUC AGA CUG AUG UUG A
miR-21 RT primer	TGG GCA CGG ACG GTA AGT AAG TCC ACC TCT CTC CAC CAT CGT CTC TAG TTC AAC AT
miR-21 RT-PCR reverse primer	GCC CGT AGC TTA TCA GAC TG
miR-155	UUA AUG CUA AUC GUG AUA GGG GU
miR-155 RT-PCR RT primer	TGG GCA CGG ACG GTA AGT AAG TCC ACC TCT CTC CAC CAT CGT CTC TAG TAC CCC TAT
miR-155 RT-PCR reverse primer	GCC CGT TAA TGC TAA TCG TG
RT-PCR universal forward primer	GGG CAC GGA CGG TAA GTA AG
MM21-1	UAG CUU AUC ACA CUG AUG UUG A
MM21-2	UAA CUU AUC ACA CUG AUG UUG A
MM21-3	UAA CUU AUC ACA CUG AUG UCG A
MM155-1	UUA AUG CUA AUC GUG AUA GGG GU
MM155-2	UUA AUG CUA AUC GUG AUA GGG GU
MM155-3	UUA AUG CUA AUC GUG AUA GGG GU

[a]:The bases in MM21-1, MM21-2 and MM21-3 that differ from those in miR-21 and MM155-1, MM155-2 and MM155-3 that differ from those in miR-155 are marked in red.

MiRNA detection procedures

The miRNA detection contained two steps. The first step is a RT process and the second step is a qRT-PCR process. The RT process was conducted by the following method. 4 μL of 5 × RT buffer, 1 μL of dNTP Mix (10 nM each), 1 μL of RNA inhibitor (40 U/μL), 1 μL of RT primer (1 μM), 1 μL of M-MLV (H-) reverse transcriptase (100 U/μL), synthetic miRNA or RNA extracted from different cell lines, tissue specimens or serum specimens and DEPC-treated water were mixed to a final volume of 20 μL. The RT was conducted under the following conditions: 45 min at 42 °C and 15 min at 70 °C. The RT products were store at −20 °C. The qRT-PCR was conducted by the following method. 5 μL of AceQ qPCR SYBR Green master mix, 0.2 μL of forward primer (10 μM), 0.2 μL of reverse primer (10 μM), 1 μL of RT product and DEPC water were mixed to a final volume of 10 μL. The qRT-PCR was conducted under the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 15 s, and 60 °C for 30 s. The fluorescence signal was detected at 60 °C. The melting curve analyses were detected by following conditions: 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s.

Results and discussion

Design of universal primer set frame and the principle of the assay

The principle of the real-time quantification of miRNA using universal primer set frame is illustrated in Scheme 1. The whole detection process includes RT and qRT-PCR two steps which are performed separately. The universal primer frame, one RT primer frame, one qRT-PCR forward primer frame and one qRT-PCR reverse primer frame, was designed by homebuilt software. The design principle of our homebuilt softwar was described in supporting information. The RT primer frame contains three parts, RT-X (7 nt), RT-Y (28 nt) and RT-Z (21 nt) and can be applied for multiplex miRNAs RT by simply changing the RT-X part