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One-step real time RT-PCR for detection of microRNAs

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ABSTRACT

Rapid and simple methods for microRNA (miRNA) detection are essential for biological research of miRNAs and clinical diagnosis. Here we describe a sensitive and specific real time RT-PCR (also RT-qPCR) method for miRNA quantification. The whole detection process including reverse transcription and PCR is performed in one PCR tube by a one-step operation on a real-time PCR system. The results display a wide linear range from 0.1 amol to 10 fmol with a detection limit of 12.6 zmol for miRNA let-7a detection. Let-7a in small RNA samples extracted from tumor cells has been successfully detected by this method. This method is cost-effective, simple and rapid, and has the advantages in the high-throughput routing assay of given miRNAs, as well as in non-model research that has less specific kits and reagents.

1. Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that play important roles in various physiological processes through the post-transcriptional regulation of gene expression [1,2]. Dysexpression of miRNAs is involved in many diseases, such as cancer [3,4], diabetes [5], immune system disease [6], muscle disorders [7], neurodegeneration [8], etc. Recently, miRNAs have been considered as a new class of biomarkers for the diagnosis of cancer and other diseases [9,10]. Therefore rapid and simple detection methods for miRNAs are imperative, not only for better understanding of the biological functions of miRNAs but also for clinical diagnosis.

Northern hybridization and microarray analysis are the classic tools for miRNA detections [6,11]. However, the sensitivity and selectivity of these methods do not satisfy the detection of the less abundant miRNAs [12,13]. In order to improve the sensitivity and specificity of miRNAs detection, many amplification methods have been reported, such as nanoparticle amplification methods [14], conjugated-polymer-based methods [15,16], modified invader assay [17], reverse transcription polymerase chain reaction (RT-PCR) [18–20], ribozyme amplification methods [21], rolling cycle amplification [22–25], and isothermal amplification [26,27]. Among these methods, the RT-PCR is the most practical method for miRNA detection. However, because the reverse transcription step for miRNAs is time consuming and usually performed at a low temperature, it is not compatible with the fast thermocycling process of PCR [18–20]. Therefore the RT-PCR for miRNA assay usually involves multiple sample processing steps. Additionally, the use of locked nucleic acids (LNA) [28], stem–loop probes [18,19,29,30], TaqMan probes [18], or ribonucleotide-modified DNA probes [31] make these methods costly and complex (indicate probe design).

In this study, we describe a one-step real time RT-PCR (also RT-qPCR) method for the detection of miRNA. This method combines the reverse transcription and PCR in a single tube; all the reagents are added together, and the reverse transcription and PCR processes are conducted consecutively on a real time PCR system.

2. Experimental

2.1. Materials and reagents

PAGE-purified DNA oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). HPLC-purified miRNAs, RNase inhibitor, Reverse Transcriptase M-MLV (RNase H$^-$), HS Taq^{TM}, RNAiso for small RNA, and diethylpyrocarbonate (DEPC)-treated water were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Chloroform and isopropanol were obtained from Beijing chemical plant (Beijing, China). SYBR Green I (20 × stock solution in dimethyl sulfoxide, 20 mg mL^{-1}) was purchased from Beijing Fanbo Biochemicals Co., (Beijing, China). TaqMan small RNA assay kit was purchased from Life Technologies Corporation. All the

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solutions for real-time RT-PCR were prepared in DEPC-treated water. The used sequences of RNA and DNA oligonucleotides are listed as follows:

Let-7a miRNA: 5′-UGAGGUAGUAGGUUGUAUAGUU-3′
Mir-122: 5′-UGGAGUGUGACAAUGGUGUUUG-3′
Primers for let-7a:
RP1: 5′-GGACGGTAGCAAGCAAAGAGTGTGAACTATACAAC-3′
RP2: 5′-GGGATTCTGGAAGATGATGATGACTGAGGTAGTAG-3′
P1: 5′-GGACGGTAGCAAGCAAAGAGTGTG-3′
P2: 5′-GGGATTCTGGAAGATGATGATGAC-3′
Primers for mir-122:
RP1-122: 5′-GGACGGTAGCAAGCAAAGAGAGAGCAAACACCATT-3′
RP2-122: 5′-GGGATTCTGGAAGATGATGATGACTGGAGTGTGAC-3′
P1-122: 5′-GGACGGTAGCAAGCAAAGAGAGAG-3′
P2: 5′-GGGATTCTGGAAGATGATGATGAC-3′
Pre-let-7a miRNA let-7a-3: 5′-GGGUGAGGUAGUAGGUUGUAUAGUUUGGGGCUCUGCCCUGCUAUGGGAUAACUAUACAAUCUACUGUCUUUCCU-3′
Forward primer for let-7a-3: 5′-TAATACGACTCACTATAGGGAGGGTGAGGTAGTAGGTTGTATAGTTTGGGGCTCTGCC-3′
Reverse primer for let-7a-3: 5′-AGGAAAGACAGTAGATTGTATAGTTATCCCATAGCAGGGCAGAGCCCCAAACTATAC-3′

2.2. Preparation of pre-let-7a miRNA with in vitro transcription reaction

Pre-let-7a miRNA (let-7a-3) is a precursor of let-7a miRNA, which is prepared by in vitro transcription reaction according to the previous literature [32]. Firstly, let-7a-3 forward primer (FP) and reverse primer (RP) are designed (please see above), in which 20 bases in the 3′-terminus of let-7a-3 FP is complementary to 20 bases in the 3′-terminus of let-7a-3 RP. 50 pmol of let-7a-3 FR and RP was mixed in a 10 μL volume of Klenow buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM Dithiothreitol (DTT), pH 8.0). The mixture was incubated at 75 °C for 5 min and slowly cooled to room temperature (~30 min) to perform the hybridization between the 20 complementary bases. After that, dNTPs (250 μM final), 5 U Klenow DNA polymerase (exo-) (NEB, Beijing, China), Klenow buffer and DEPC-treated deionized water were added in the mixture to give a final volume of 20 μL. With incubation at 37 °C for 1 h, the let-7a-3 FP and RP performed the extension reaction at their 3′-termini, to form a double stranded (ds) DNA. The reaction mixture was heated at 75 °C for 20 min to inactivate the Klenow DNA polymerase and then slowly cooled to room temperature for dsDNA annealing. The dsDNA consisted of the T7 promoter, GGGA spacer and let-7a-3 specific sequence (from 5′to 3′-terminus in the upper strand).

A volume of 20 μL of the dsDNA solution was added into 30 μL of in vitro transcription buffer (containing 2 mM NTPs, 40 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, pH 7.9), 100 U Ribonuclease inhibitor, and 80 U T7 RNA polymerase (Thermo scientific, Beijing, China). The in vitro transcription reaction was performed at 37 °C for 4 h to produce the pre-let-7a miRNA (let-7a-3). After that, the DNA in the reaction mixture was digested for 30 min by adding 5U of RNase-Free DNase I (TaKaRa, Dalian, China) and then the mixture was purified with Phenol/chloroform extraction. The final products of let-7a-3 were confirmed by the electrophoresis analysis with 4% Agarose gel, and the concentration was determined from the absorption at 260 nm with SpectraMax M5 (Molecular Devices, CA, USA).

2.3. Small RNA extraction from cells

All cells were routinely grown in a humidified incubator at 37 °C with 5% CO_2. 7721 (human hepatocarcinoma), PC3, T47D, K562 (leukemia) cells were purchased from Cell Culture Center of Institute of Basic Medical Sciences (Chinese Academy of Medical Sciences, Beijing, China), and cells were grown in RPMI 1640 (Gibico) medium supplemented with 10% FBS, and 1% penicillin/streptomycin for 48 h.

RNAiso for small RNA was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Small RNA extraction was carried out in accordance with the instructions by the manufacturer. The OD_{260}/OD_{280} of small RNA extracted from 7721, PC3, T47D, and K562 cells are 1.8, 1.8, 1.9 and 1.9 respectively, which indicate that the small RNAs are in good quality.

2.4. Experimental procedures for miRNA detection

1 μL of 5 × M-MLV Buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM Dithiothreitol), 1 μL of 10 × PCR Buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.1 μL of 2 μM primer RP1, 0.1 μL of 2 μM primer RP2, 0.1 μL of 10 μM primer P1, 0.1 μL of 10 μM primer P2, 0.2 μL of 20 × SYBR Green I, 4 U RNase inhibitor, 20 U Reverse Transcriptase M-MLV (RNase H^-), 1 U HS Taq™, miRNA, synthetic RNA or small RNA extracted from cells and DEPC-treated water were mixed to a final volume of 10 μL. The real-time RT-PCR assay was conducted under the following conditions: Stage 1: 3 min at 45 °C, 5 min at 37 °C; Stage 2: 30 s at 95 °C, 30 s at 37 °C, 30 s at 60 °C; and Stage 3: 40 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C. And the real-time fluorescence intensity was monitored at each cycle of the third stage. StepOne Real-Time PCR System (Applied Biosystems, USA) was used to perform the reaction.

3. Results and discussion

3.1. Principle of the one-step real time RT-PCR

The principle of the one-step real time RT-PCR is illustrated in Fig. 1. The whole detection procedure requires four primers: RP1, RP2, P1 and P2. RP1 contains the P1 and a 11-base sequence R1c that is complimentary to the 3′-end of target miRNA. RP2 contains P2 and a 11-base sequence R2 that is same with the 5′-end of target miRNA. The total thermocycling program includes three stages. Stage 1 is the reverse transcription process. In this stage, the R1c part of RP1 hybridizes with target miRNA, and then is extended in the presence of Reverse Transcriptase M-MLV (RNase H^-) and dNTPs. Since the melting temperature (T_m) of an 11-base sequence is near 37 °C, this stage is conducted at 37 °C. In Stage 2, after denaturing at 95 °C, the R2 part of RP2 hybridizes with the cDNA of miRNA at 37 °C, and both sequences

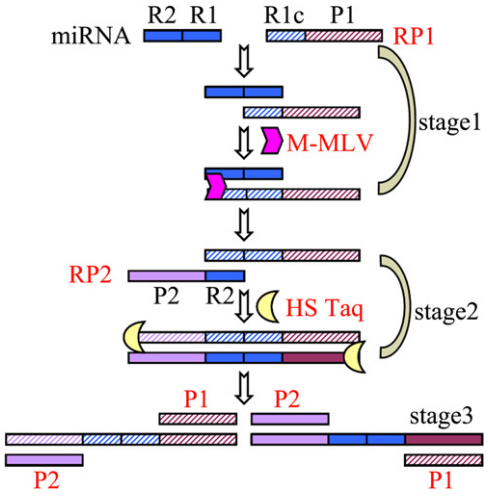

Fig. 1. Schematic illustration of the one-step real time RT-PCR detection of miRNA.