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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

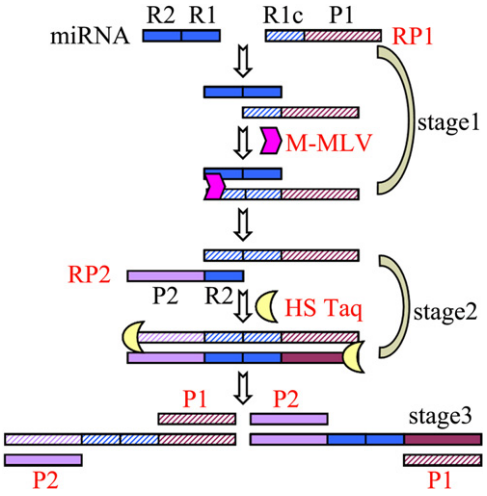

Fig. 1. Schematic illustration of the one-step real time RT-PCR detection of miRNA.

are extended in the presence of hot-start Taq polymerase (HS Taq) and dNTPs at 60 °C. Stage 3 is a conventional PCR process with the primers P1 and P2. The amplification of cDNA is monitored in the real time PCR system using SYBR green I.

3.2. Sensitivity of the proposed assay

Synthetic human miRNAs, let-7a and mir-122 were used as model miRNAs to optimize the experimental condition (see Figs. S1–S9). In order to avoid the formation of primer dimer and hairpin of RP1 and RP2, the primer P1 for let-7a was designed to have a two-base difference from that for mir-122 (P1-122), but primer P2 for both miRNAs was identical. The optimization experiments showed that the following conditions were optimal for both let-7a and mir-122 assay: 20 nM RP1/RP2, 20 unit M-MLV, 1 unit HS Taq (TaKaRa), 100 nM P1/P2; the thermocycling program: 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. Under the optimized conditions, the dynamic range and sensitivity of the one-step real time RT-PCR assay for let-7a and mir-122 were investigated (Figs. 2 and 3). Excellent linearities between the C_T values and the logarithms (lg) of target miRNA amount for both miRNAs were obtained in the range from 0.1 amol to 10 fmol. The detection limit of this method for let-7a is 12.6 zmol, and for mir-122 is 30.1 zmol. Repeated experiments showed good reproducibility of this assay (see Fig. S10). The gel electrophoresis assay (Fig. 2c) showed that only the amplification products of let-7a (70 bp) were observed after 32 cycles of amplification. The nonspecific amplification products were found slightly shorter than that of let-7a after 50 cycles of amplification.

3.3. Specificity of the proposed assay

To evaluate the specificity of the proposed miRNA assay, let-7a and mir-122 were amplified respectively with both sets of primers. As shown in Fig. 4a, only mir-122 can be amplified with mir-122 specific primers; meanwhile, an equivalent amount of let-7a did not produce obvious signal compared to the blank, the ΔC_T ($C_{Tmir\text{-}122} - C_{Tlet\text{-}7a}$) reached 12.3. The same results were obtained for the assay of let-7a (Fig. 4b), only let-7a specific primers produced well defined signal and the nonspecific signal from an equivalent amount of mir-122 sample was identical to the blank. These results suggest that the proposed method has high specificity for diferent miRNAs. To further evaluate the discrimination ability to highly homologous sequences, the let-7 miRNA family members (let-7a–e, Fig. 4c) were measured using the primers for let-7a. These sequences have a single or a few bases difference. Their expression levels are closely associated with cell development and human cancers [33]. As shown in Fig. 4c, the let-7a could be clearly discriminated from other let-7 miRNAs. There is a two-base difference among let-7a, let-7b and let-7e; and a three-base difference between let-7a and let-7d. The nonspecific amplification of let-7b, let-7d and let-7e was very little (8.1%, 0.1% and 0.08%). The non-specific amplification of let-7c was relatively high (29%), which may be due to the single base change of A (let-7a) to G (let-7c) because the interaction energy between T–A and T–G is similar [34]. This specificity of the proposed miRNA assay is comparable to that of many other miRNA assays [26,27,31].

3.4. Discrimination of mature miRNA against pre-miRNA

Since mature miRNAs are derived from the precursor (Pre-miRNA) (~70 nt), the Pre-miRNA may disturb the assay of mature

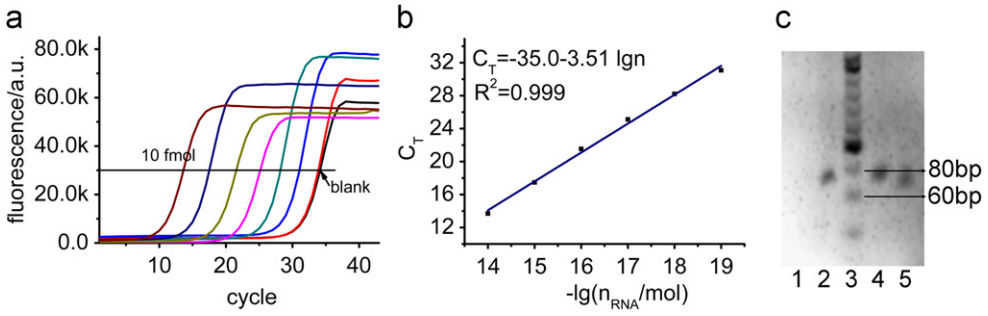

Fig. 2. Dynamic range and sensitivity of the let-7a assay. (a) Amplification plot of synthetic let-7a. Except blank, from right to left, the concentration of let-7a successively is 0.01 amol, 0.1 amol, 1.0 amol, 10 amol, 0.1 fmol, 1.0 fmol and 10 fmol correspondingly. Blank: amplification mixture without let-7a. (b) The relationship between C_T and lg of let-7a amount (mol). (c) The gel electrophoresis analysis (3% agarose) of the amplification products. lane 1: blank 32 cycles, lane 2: 10 fmol let-7a 32 cycles, lane 3: marker, lane 4: 10 fmol let-7a 50 cycles, and lane 5: blank 50 cycles.

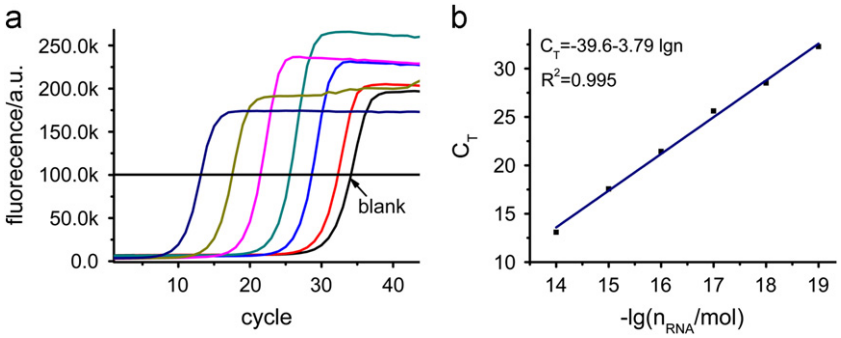

Fig. 3. Dynamic range and sensitivity of the let-7a assay. (a) Amplification plot of synthetic mir-122. Except blank, from right to left, the concentration of mir-122 successively is 0.1 amol, 1.0 amol, 10 amol, 0.1 fmol, 1.0 fmol, 10 fmol correspondingly. Blank: amplification mixture without let-7a. (b) The relationship between C_T and lg of mir-122 amount (mol).

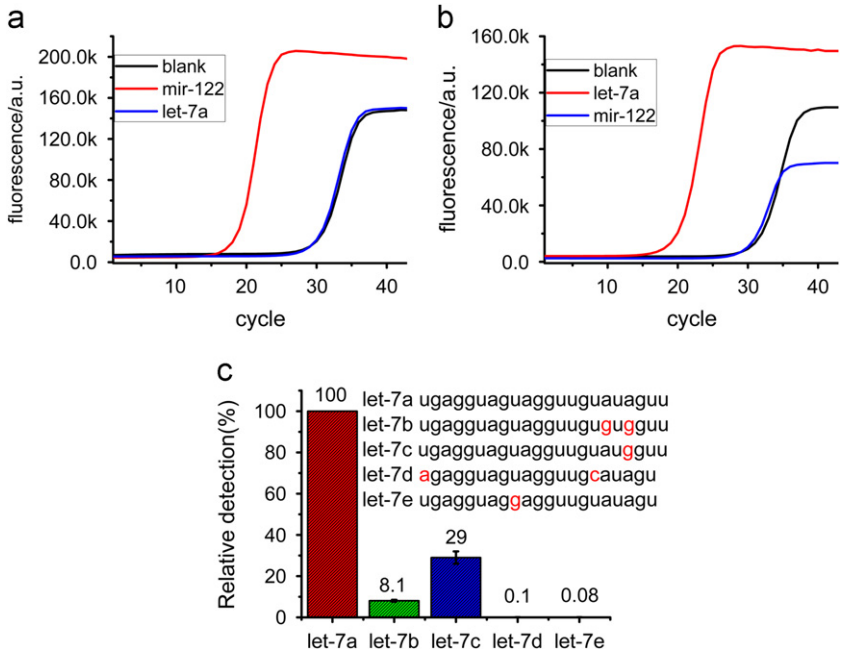

Fig. 4. One-step real-time RT-PCR detection of mir-122 and let-7a (0.1 fmol) by using (a) mir-122 specific primers and (b) let-7a specific primers. (c) Discrimination of human let-7 b-e from let-7a by real time RT-PCR assay. Each of the let-7 miRNA was 1 fmol. The relative detection of let-7a was defined as 100% and the relative detection of other miRNA was calculated by $1/1.9^{\Delta C_T}$, in which $\Delta C_T = C_{T,\text{other miRNA}} C_{T,\text{let-7a}}$.The results were average values with three repetitive measurements.

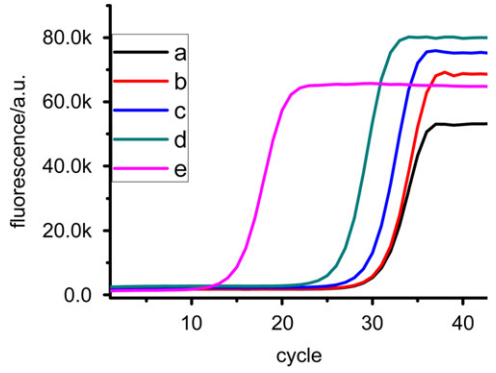

Fig. 5. Amplification plots of let-7a and let-7a-3 with the one-step real time RT-PCR assay: (a) blank, (b) 10 amol let-7a-3, (c) 0.1 fmol let-7a-3, (d) 1 fmol let-7a-3, and (e) 1 fmol let-7a.

miRNA. To investigate the capability of the one-step real-time RT-PCR assay in discriminating mature against precursor miRNAs, let-7a and its pre-miRNA, let-7a-3, were measured by this method. The experimental results showed that let-7a-3 only produced negligible PCR amplification signals when its amount is less than 10 amol. By detecting 1 fmol of let-7a and let-7a-3, the let-7a-3 was detected 10.9 cycles later than let-7a, corresponding to 0.05% nonspecific detection (Fig. 5). This high discrimination capacity may be due to the stem–loop structure of the pre-miRNA that reduces the primer hybridization to let-7a-3 under the condition of reverse transcription. These results indicate that this one-step real-time RT-PCR assay has high specificity for mature miRNA detection against the corresponding pre-miRNAs.

3.5. Detection of let-7a in small RNAs extracted from cells

In order to assess the practicability of the proposed method, the small RNAs extracted from 7721, PC3, T47D and K562 cells were applied to determine the amount of let-7a. As shown in Fig. 6, good linear relationship between C_T values and the small RNA inputs was observed in the range of 100 pg–100 ng. The contents of let-7a in small RNA sample of 7721, PC3, T47D and K562 cells were found to be 8.95, 5.13, 7.94 and 1.41 amol per ng respectively. In order to demonstrate the accuracy of the method, the same small RNA samples were detected by TaqMan MicroRNA Assay (Catalogue number 4427975 and 4366596, Life Technologies) (see Fig. S11). The contents of let-7a in small RNA sample of 7721, PC3, T47D and K562 were found to be 9.11, 4.54, 7.56 and 1.25 amol per ng. These results were coincident with that obtained by the proposed one-step real time RT-PCR assay, indicating the good practicability of the one-step real time RT-PCR assay for quantification of miRNAs in real world samples.

3.6. Discussion

The above results have demonstrated that the proposed real-time RT-PCR assay can work well for mature miRNA quantification. The biggest merit of this method is that the whole detection procedure is performed in one PCR tube with one-step operation. In the previous RT-PCR method for miRNA assay, the reverse transcription process is usually performed at different temperatures for a relatively long time (for example, stem–loop RT-PCR method needs: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C),[18] which have to be performed separately before RT-PCR amplifications. In the proposed method, all the primers and enzymes are added together into a PCR-tube, the three stages of the whole assay are performed automatically in a real-time PCR system. This single-step operation not only simplifies the working process but also decreases the chance of contamination, which makes this method more suitable for high throughput assay. The total reverse transcription stage is only 8 min, which is much shorter than that of the previous method. The Stage 2 is a one-cycle PCR with annealing at 37 °C and extension at 60 °C, which produces a duplex DNA containing primers P1, P2 and DNA version of the target miRNA. Then the products of Stage 2 are amplified by

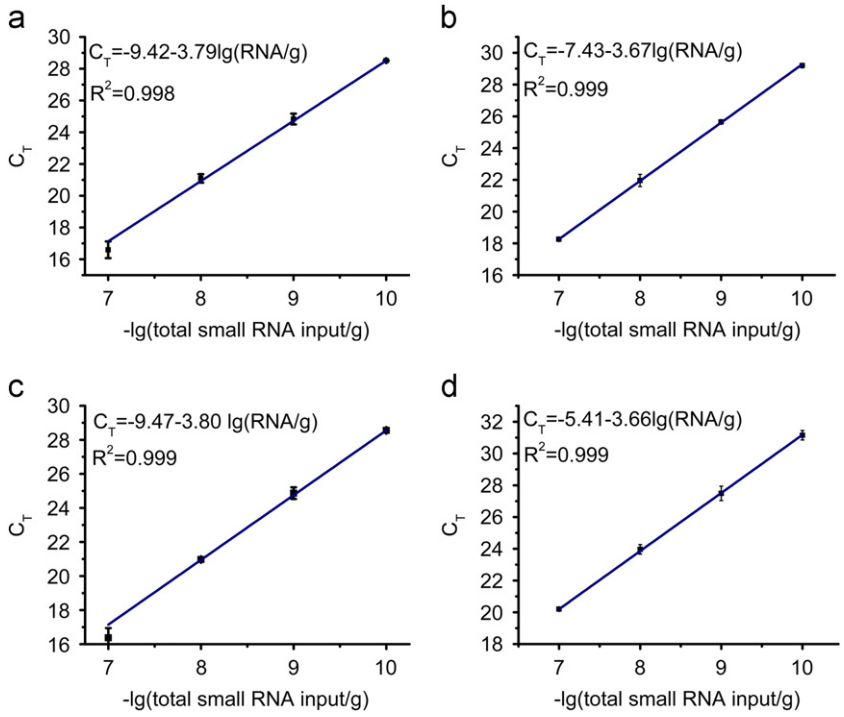

Fig. 6. The relationship between C_T values and inputs of small RNAs. Small RNAs were extracted from 7721 (a), PC3 (b), T47D (c) and K562 (d) cells.

real-time PCR using primers P1 and P2. Since primers P1 and P2 would not work in Stage 1 and Stage 2, it can be added to the reaction system together with the reagents for reverse transcription. Because primer RP1 for reverse transcription and primer RP2 for Stage 2 are not required in the PCR amplification stage, the amounts of them can be greatly reduced, which may reduce the nonspecific reaction in the first two stages. In order to reduce the nonspecific amplification, hot-start Taq polymerase is used for PCR amplification. Additionally, this proposed method does not require any modified primers or labeled DNA probes, and only requires two common and cheap enzymes, and SYBR green I for fluorescence detection, which significantly reduce the cost of the assay (see Table S1). The primer design is very easy, because primers P1 and P2 can be used for different miRNA assays after slight modification based on the target miRNA sequences.

4. Conclusions

In summary, we have proposed a one-step real time RT-PCR method for the quantification of miRNAs. The proposed miRNA assay exhibits high selectivity, high sensitivity, and can detect as low as 12.6 zmol miRNA. It has been successfully used for miRNA detection in small RNA samples extracted from tumor cells. The low cost, easy design, simple operation and high speed, make this method have 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.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.02.028.

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