



## ARTICLE



# Long transcripts minus touchdown qPCR (LTMT-qPCR): a simplified and convenient method for the screening and quantification of microRNA profiles

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Due to the short length and differences in abundance of microRNAs, microRNA profile screening and quantification is challenging. In this study, we found that size selection magnetic beads could be employed to easily and efficiently remove long RNA transcripts. After removing the long transcripts, the remaining small RNAs could be concentrated and then reverse-transcribed using universal stem-loop primers (USLP), with six randomized nucleotides at the 3' end region. The efficiency of reverse transcription decreased when the number of randomized nucleotides was reduced. In addition, we found that touchdown qPCR improved microRNA profile detection, with lower  $C_T$  values and better detection efficiency than the regular qPCR protocol, especially for those low-abundance microRNAs. Finally, we incorporated these observations to create a new protocol we named long transcripts minus touchdown qPCR (LTMT-qPCR). We performed a side-by-side comparison of LTMT with USLP and traditional stem-loop primer (TSLP) protocols. We found that LTMT has higher detection efficiency than USLP, especially for the detection of low-abundance microRNAs. Although LTMT was equivalent to TSLP in terms of microRNA profile detection, LTMT is more convenient, user-friendly, and cost-effective. Taken together, the present data indicate that LTMT is a simple, rapid, and user-friendly approach that has higher precision, accuracy, and sensitivity than the previously described methods, making it more suitable for microRNA profile screening and quantification.

*Laboratory Investigation* (2021) 101:1618–1626; <https://doi.org/10.1038/s41374-021-00648-9>

## INTRODUCTION

MicroRNAs are small (~22 nt, ranging from 18 to 25 nt in length) non-coding RNAs that regulate gene expression by catalyzing the cleavage of messenger RNA (mRNA) or repressing mRNA translation<sup>1–5</sup>. Derived from genome-encoded stem-loop precursors, microRNAs function through RNA-induced silencing complex-mediated binding to their target mRNAs by base pairing, mostly to the 3' un-translated region<sup>2–4</sup>. They play significant roles in several cellular and pathological processes, including cell proliferation, death, development and differentiation, metabolism, and communication<sup>6–9</sup>. As a result of these diverse functions, microRNAs participate in the development and progression of many different human diseases, including cancer<sup>10–15</sup>. To date, more than 2000 microRNAs have been discovered, and the abundance of different microRNAs (i.e., let-7a-5p) in different cells and tissues varies from 0 to  $1.4 \times 10^5$  reads per million<sup>16</sup>. Based on this difference, microRNAs can be divided into low-, medium-, and high-abundance microRNAs.

Although many methods for detecting microRNAs have been developed, such as northern blotting<sup>17</sup>, in situ hybridization<sup>18,19</sup>, microRNA arrays<sup>20,21</sup>, bead arrays<sup>22</sup>, and next-generation sequencing (NGS)<sup>23</sup>, reverse transcription-quantitative polymerase chain

reaction is still the most commonly used method due to its advantages in sensitivity, flexibility, and simplicity<sup>24–28</sup>. However, due to their short lengths and differences in abundance, microRNA expression profile screening and quantification is still challenging, with small RNA recycling, small RNA reverse transcription, and small RNA qPCR quantification being the critical steps<sup>29</sup>.

The abundance of long transcripts, such as 28S and 18S ribosomal RNA, lncRNA, and mRNA, within the total cellular RNA hinders the reverse transcription of microRNAs by wasting primers and the inclusion of microRNA sequencing of the transcripts (e.g., primary microRNA). The impact of these long transcripts is worse when universal primers are used. To remove these long transcripts, PAGE gel recycling is commonly used to obtain more targeted products<sup>30</sup>, but it is time-consuming and prone to contamination. Therefore, it is more frequently used for microRNA array and microRNA NGS library construction, rather than routine studies<sup>31</sup>. PAGE gel recycling is impractical for qPCR-based screening, so it is necessary to develop a rapid and user-friendly method to remove these long transcripts ( $\geq 200$  nt) so the product can be used for qPCR.

Size selection magnetic beads (SSMBs) are magnetic carriers coated with negatively charged carboxyl molecules on the surface

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Received: 16 May 2021 Revised: 17 July 2021 Accepted: 19 July 2021

Published online: 10 August 2021

and suspended in a special buffer containing saturated polyethylene glycol (PEG) and high concentration of salt ( $\text{Na}^+$ ), which helps the nucleic acids to form large random coils and cause them to be aggregate and precipitate. The precipitated nucleic acids are captured by the coated magnetic beads and are separated by a magnetic stand. However, if the SSMBs were mixed with an aqueous solution such as  $\text{ddH}_2\text{O}$ , the buffer with PEG and  $\text{Na}^+$  will be diluted. Diluted buffer only removes large size nucleic acids and small fragments will turn back into solution again. Therefore, beads (containing PEG and  $\text{Na}^+$ )-to-sample (DNA or RNA dissolved in  $\text{ddH}_2\text{O}$ ) volumetric ratio (B/S) will determine the size of DNA/RNA fragments to be retained in the solution or adsorbed by the beads. For the final step, when SSMBs were suspended in aqueous solution without any PEG and  $\text{Na}^+$ , all the nucleic acids on the SSMBs will be released, thus separating the nucleic acids by size. SSMBs are well developed as a widely used strategy for DNA/RNA size selection instead of gel recycling in the NGS library preparation. In our prior work, we optimized and developed a user-friendly protocol for removing contaminating RNA from pDNA using SSMBs, which separated the pDNA from contaminating RNA based on their differences in size<sup>32</sup>. As there is also a large size difference between long transcripts ( $\geq 200$  nt) and microRNA (18–25 nt), we hypothesized that the SSMBs could also be employed to remove long transcript RNA from total RNA.

Another crucial factor for microRNA determination is reverse transcription, as microRNAs are only about 22 nt in length, and the reverse-transcribing primer cannot easily capture the target microRNA. Various methods have been used to fix this problem, such as sequencing-specific primers with a fixed linear tag or a stem-loop structure<sup>24–28,33–35</sup>. In most of the studies and in our unpublished data, the use of a stem-loop primer with the last six nucleotides perfectly reverse-complementary to the 3' end region of the microRNA appeared to be more specific than the use of linear tags<sup>24,26–29,33–35</sup>. However, if this method is used, a specific stem-loop primer is needed for each microRNA, and an additional reverse transcription reaction may be required to detect a new microRNA. To optimize this strategy, Yang et al. developed a method called “universal stem-loop primer (USLP)” that uses eight random nucleotides instead of a specific sequence at the 3' end of the traditional stem-loop primer (TSLP), permitting reverse transcription of all of the microRNAs simultaneously in a single reaction<sup>35</sup>. This method reduced the cost of primers by 75%, and reduced the testing time by 60%, without removing long transcripts. Unfortunately, low-abundance microRNAs are difficult to be detected by USLP in the presence of long transcripts. Long transcript removal is therefore essential to improve the sensitivity and to avoid the USLP being used up by the long transcripts. After long transcript removal, the residual small RNAs can be concentrated to ensure that the low-abundance microRNAs can be detected.

Fluorescence-based real-time qPCR, including fluorophore-labeled probe qPCR and dye staining (e.g., SYBR green)-based qPCR, is another pivotal step for microRNA quantification. The PCR amplification efficiency and specificity determines the sensitivity and accuracy of detection. Previously, Zhang et al. developed a novel and effective touchdown qPCR (Td qPCR) protocol with a four-cycle touchdown stage before the regular qPCR (Reg qPCR) amplification stage<sup>36</sup>. In their study, using the same cDNA templates, Td qPCR reduced the average  $C_T$  value by five cycles on average and improved the PCR efficiency compared with Reg qPCR, thus increasing the detection sensitivity, especially for the less abundant genes. As most microRNAs are expressed at low levels, we decide to employ this protocol for microRNA profile detection.

By combining these innovations in microRNA detection, we developed a new protocol named long transcripts minus touchdown qPCR (LTMT-qPCR). Following validation of the method, we evaluated the microRNA expression profile in SJS1 cells treated

with nutlin3A or DMSO control. The results presented herein provide evidence that LTMT-qPCR is a simple, rapid, and user-friendly method with high precision, accuracy, and sensitivity, making it suitable for microRNA profile screening and quantification.

## MATERIAL AND METHODS

### Cell culture and chemicals

SJS1 and HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% FBS (Gemini Bio-Products), as described previously<sup>32,37,38</sup>. All DNA oligos and RNA products were synthesized by BGI Genomics in China, and the sequences are listed in Supplementary Data 1.

### Total RNA extraction and long transcript RNA removal by size selection magnetic beads (SSMBs)

The total RNA was extracted with the NucleoZOL RNA Isolation kit (Takara Bio USA, Mountain View, CA) according to the manufacturer's instructions and as described in previous studies<sup>38,39</sup>. The commercially available SSMBs Mag-Bind<sup>®</sup> TotalPure NGS (Omega Bio-tek, Inc., Norcross, GA) were used for the study in a manner similar to the previously reported plasmid DNA purification protocol<sup>32</sup>. Briefly, the total RNA (approximately 5  $\mu\text{g}$  RNA dissolved in 20  $\mu\text{l}$   $\text{ddH}_2\text{O}$ ) and the Mag-Bind beads were mixed at a vol/vol ratio of 1:1 (RNA: Beads) and then mixed vigorously. The DNA-beads mixture was incubated at room temperature for 10 min. The mixture was separated by a magnet stand and the small RNA (<200 nt)-containing supernatant was collected, while long transcripts (>200 nt)-bound beads were discarded. Next, the collected small RNA was purified using the phenol/chloroform method, followed by ethanol precipitation. After precipitation, the small RNA was dissolved in 20  $\mu\text{l}$   $\text{ddH}_2\text{O}$  and used for the reverse transcription reaction or stored at  $-80^\circ\text{C}$ .

### RNA bio-analyzer analysis

After long transcript removal, the small transcript RNA was assessed with an Agilent 2100 Bioanalyzer (Santa Clara, CA). Briefly, purified small RNA samples (1.0  $\mu\text{l}$ ) were loaded onto the Bioanalyzer RNA Nano Chips, along with size marker and control RNA. The chip was subjected to electrophoresis according to the manufacturer's instructions. The integrity and quantity of RNA samples were assessed using both gel images and electropherograms.

### MicroRNA reverse transcription reaction

A 10  $\mu\text{l}$  aliquot of purified total RNA or long transcripts minus RNA were mixed with different 2.0  $\mu\text{l}$  stem-loop oligonucleotides (stem-loop-SP, stem-loop-6N, stem-loop-4N, stem-loop-3N, or stem-loop-2N, 1.0  $\mu\text{g}/\mu\text{l}$ ) and annealed at  $70^\circ\text{C}$  for 5 min. After the samples were cooled down on ice, 0.5  $\mu\text{l}$  of RNase Inhibitor, 2.0  $\mu\text{l}$  of  $10\times$  RT buffer (NEB), 2.0  $\mu\text{l}$  of 10 mM dNTPs, 0.5  $\mu\text{l}$  of M-MLV reverse transcriptase, and 3.0  $\mu\text{l}$  RNase-free  $\text{ddH}_2\text{O}$  were added (for a 20  $\mu\text{l}$  reaction). The reaction was kept at  $25^\circ\text{C}$  for 10 min, and then  $37^\circ\text{C}$  for 30 min.

### Reg qPCR and Td qPCR

To increase the annealing temperature, the first 17 bp of the microRNAs with “AGCC” at the 5' were used as the microRNA qPCR forward primer, and a part of the stem-loop structure “5'-GTGACAGGTCGAGGTCGAG-3'” was used as the common reverse primer. The 5S and 5.8S transcripts were used as reference genes<sup>40</sup>. The qPCR reaction system was set up as described in the manufacturer's instructions and was run on the CFX-Connect (Bio-Rad)<sup>36</sup>. The Reg qPCR program was as follows:  $95^\circ\text{C} \times 3'$  for one cycle;  $95^\circ\text{C} \times 20''$ ,  $55^\circ\text{C} \times 10''$ ,  $70^\circ\text{C} \times 1''$ , followed by plate reading, for 40 cycles. The Td qPCR program was as follows:  $95^\circ\text{C} \times 3'$  for one cycle;  $95^\circ\text{C} \times 20''$ ,  $66^\circ\text{C} \times 10''$ , for four cycles by decreasing  $3^\circ\text{C}$  per cycle;  $95^\circ\text{C} \times 20''$ ,  $55^\circ\text{C} \times 10''$ ,  $70^\circ\text{C} \times 1''$ , followed by plate reading, for 40 cycles<sup>36</sup>. A five-fold serial dilution was performed to determine the amplification efficiency for Reg qPCR and Td qPCR. The negative control used  $\text{ddH}_2\text{O}$  as the template.

### Dicer-mediated gene silencing

The SJS1 cells were infected with pSEB-si-ctrl- or pSEB-si-Dicer-containing retrovirus supernatants, which were packaged as described<sup>37,39</sup>. In brief, the pSEB-si-ctrl or pSEB-si-Dicer plasmids with pAmpho plasmids were

packaged in 293PA cells, and the supernatants were collected at 24, 36, 48, and 72 h after transfection. The collected supernatants were filtered and used to infect SJS A1 cells. The infected cells were screened with Blasticidin for cells with stable Dicer-mediated gene silencing.

### MicroRNA profile detection by LTMT, TSLP, and USLP methods

For the LTMT method, the long transcripts of total RNA were removed as described above. The purified small transcripts were reverse-transcribed by the stem-loop-6N primers. After the cDNA was diluted, the microRNA profile was determined by Td qPCR. For the TSLP method, the total RNA without long transcript removal was reverse-transcribed using the TSLP (stem-loop-SP) primer mixture, which is the reverse complement of the last six nucleotides of the target microRNA. After the cDNA was diluted the same number of times as in the LTMT method, the microRNA profile was detected by Reg qPCR. For the USLP methods, the total RNA without long transcript removal was reverse-transcribed using the USLP primer. The resulting cDNAs were diluted, and the microRNAs were screened and quantified by Reg qPCR. To make it possible to compare the efficiency of these methods side-by-side, the total starting RNA was the same.

### Analysis of microRNA expression panels

The microRNA expression was detected by LTMT, TSLP, and USLP methods. The cycle threshold ( $C_T$ ) values were analyzed and graphed. The log (fold change) value was used to make a heatmap.

### Statistical analysis

All of the quantitative studies were done in triplicate. The significance of differences between groups was determined using Statistical Product and Service Solutions (SPSS) software. A  $P$  value less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Long transcripts ( $\geq 200$ nt) were depleted completely using size selection magnetic beads (SSMBs)

In our prior work, we developed and optimized a user-friendly protocol for removing contaminating RNA from pDNA using SSMBs based on the size difference between pDNA and contaminating RNA<sup>32</sup>. As there is also a large size difference between long RNA transcripts (such as 28S RNA, 18S RNA, mRNA, and lncRNA) and microRNA, we hypothesize that the SSMBs could be employed to remove long RNA transcripts from total RNA as well. We developed and optimized a long RNA transcripts removal protocol (Fig. 1A). In brief, total RNA and Magbeads were mixed well at a ratio of 1:1 (v/v, RNA: Beads) at room temperature for 10 min (Fig. 1Aa). The mixture was then separated by a magnet (Fig. 1Ab). The short transcripts (<200 nt) remaining in the supernatant were retained (Fig. 1Ac) while the long transcripts ( $\geq 200$  nt) on the beads were discarded (Fig. 1Ad). The short transcripts in the supernatant were purified using the phenol/chloroform method, followed by ethanol precipitation, and then the short RNA was processed for reverse transcription (Fig. 1Ae).

To optimize and assess the efficiency of long transcript RNA removal, we mixed the total RNA:Magbeads at different volume ratios (5:4, 1:1, 1:2, and 1:3). After depleting the long transcripts, the remaining small RNAs were analyzed with an Agilent 2100 Bioanalyzer. One tenth of total RNA without treatment was used as an input control. As shown in the representative gel image (Fig. 1B) and electropherograms (Fig. 1C), the RNA:Magbeads volume ratios ranging from 5:4 to 1:3 were able to remove long transcripts (>200 nt) efficiently, indicating that SSMBs are able to remove the large transcripts easily and with a large flexible range. In order to control the amount of contaminating RNA present in the supernatant and to reduce experimental costs, we suggest using a beads-to-sample volumetric ratio of 1:1.

The microRNAs were extracted from the supernatant and phenol/chloroform purification was performed. We selected 13 microRNAs at random and 5s RNA to assess their RNA loss after

long transcript removal and phenol/chloroform purification. It is interesting to note that there is only a slight non-specific loss of these small RNAs (Fig. 1D). This indicates that long transcript removal method is a powerful tool to remove the long transcripts from total RNA with limited loss of small RNA.

### The number of randomized deoxyribonucleotides at the end of stem-loop primer affects the efficiency of microRNA reverse transcription

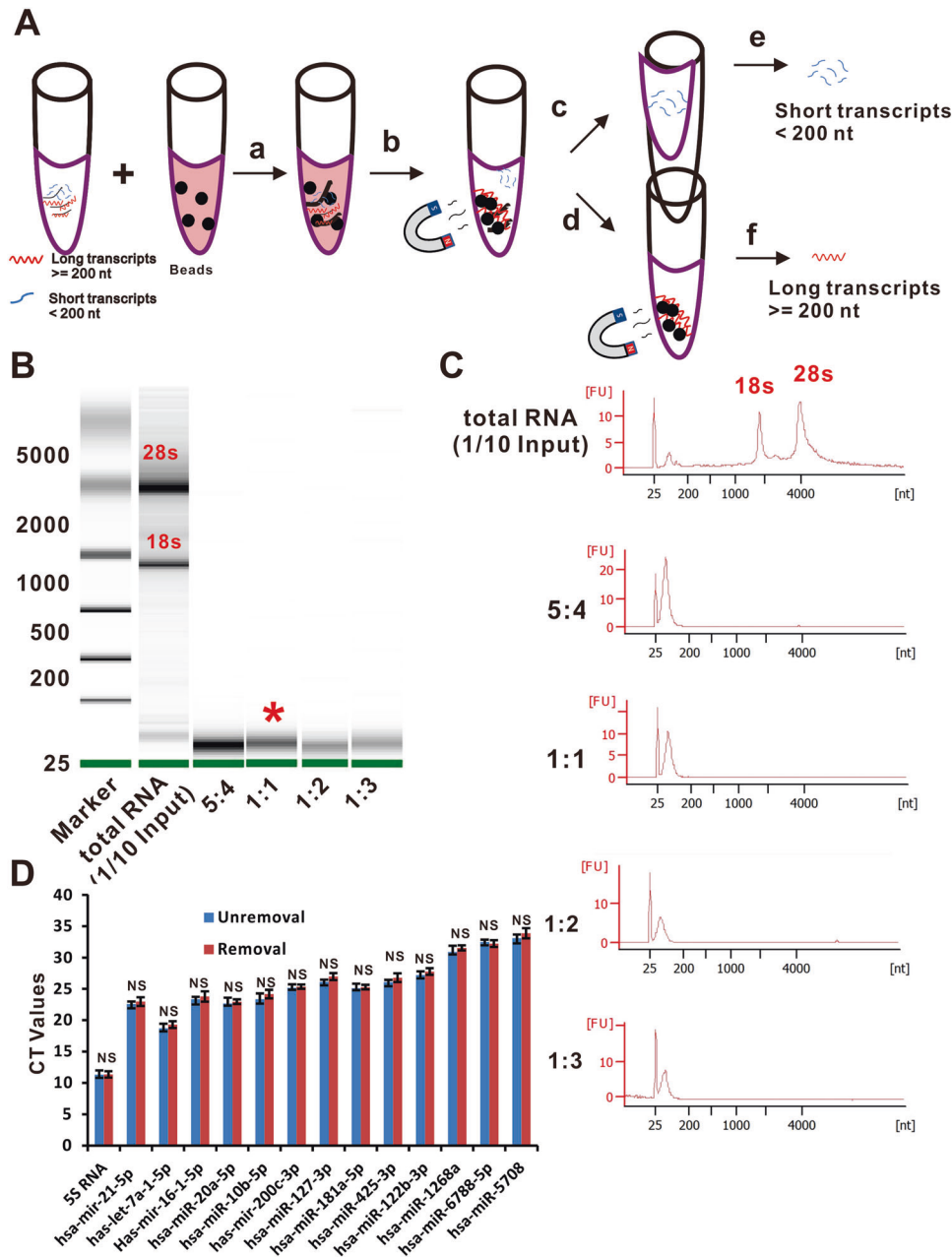
After depleting the long transcript RNA from total RNA, the mixture containing microRNAs was processed to initiate the reverse transcription reaction. A primer with a stem-loop structure at the tail of the 5' region is a commonly used reverse primer<sup>24</sup>. However, this method requires a specific TSLP primer for each microRNA. Therefore, the TSLP method is inconvenient and requires many resources. USLP with randomized deoxyribonucleotides at the 3' end region can reverse transcribe all microRNAs simultaneously, greatly improving the efficiency of reverse transcription. We designed stem-loop primers with different randomized deoxyribonucleotides (stem-loop-2N, stem-loop-3N, stem-loop-4N, stem-loop-6N) and compared them with the TSLP (stem-loop-SP) (Fig. 2A). To assess the reverse transcription efficiency among these primers, the  $C_T$  values of 5S RNA, hsa-mir-181a-5p, hsa-mir-193a-5p, and hsa-mir-6788-5p were evaluated, as these targets represented different abundances of short transcripts. As shown in Fig. 2B, C, the  $C_T$  values of 5S RNA, hsa-mir-181a-5p, hsa-mir-193a-5p, and hsa-mir-6788-5p in the stem-loop-6N group matched the values in the stem-loop-SP group, indicating high reverse transcription efficiency. However, smaller numbers of randomized deoxyribonucleotides in the end of the reverse primer resulted in higher  $C_T$  values.

Using TSLP as a control, the relative expression of these microRNAs reverse transcribed using stem-loop-6N was more stable than using stem-loop-2N, stem-loop-3N, and stem-loop-4N. This may be due to the lower stability of the structures formed between randomized deoxyribonucleotides and microRNA. In summary, stem-loop-6N is an ideal USLP for microRNA reverse transcription reactions.

### Td qPCR improved microRNA profile detection, with lower $C_T$ values and better detection efficiency, especially for low-abundance microRNAs

Zhang et al. optimized the Td qPCR program, which improved the PCR amplification efficiency and increased the detection sensitivity, especially for those low-abundance transcripts<sup>36</sup>. As most microRNAs are expressed at low levels, we tested whether Td qPCR could improve the microRNA profile detection. We randomly choose three microRNAs (let-7a-1, hsa-mir-10b, and hsa-mir-223) to represent high-, moderate-, and low-abundance microRNAs, respectively. Serial dilutions of the same cDNA samples were used to perform Reg qPCR and Td qPCR. The  $C_T$  values of let-7a-1 and hsa-mir-10b detected with Td qPCR were lower than with Reg qPCR (Fig. 3Aa–c, Ba–c). From the SYBR green fluorescence of hsa-mir-223, which is a low-abundance microRNA, the amplification curve of Reg qPCR seemed more diffuse, while in Td qPCR, it remained concentrated (Fig. 3Ca, b). The graphed hsa-mir-223  $C_T$  values in Td qPCR were lower than in Reg qPCR (Fig. 3Cc). These data suggest that Td qPCR decreases the  $C_T$  values for microRNA detection, especially for low-abundance microRNAs.

We then used siRNA to silence the expression of Dicer, which is one of the key processors of microRNAs, and detected the microRNA expression profile with Td qPCR and Reg qPCR using the same cDNA. We found that the fold change detected by Td qPCR was more significant than detected by the Reg qPCR protocol (Fig. 3D). These findings further demonstrated that the Td qPCR protocol is more efficient in detecting microRNA expression, especially for low-abundance microRNA detection.

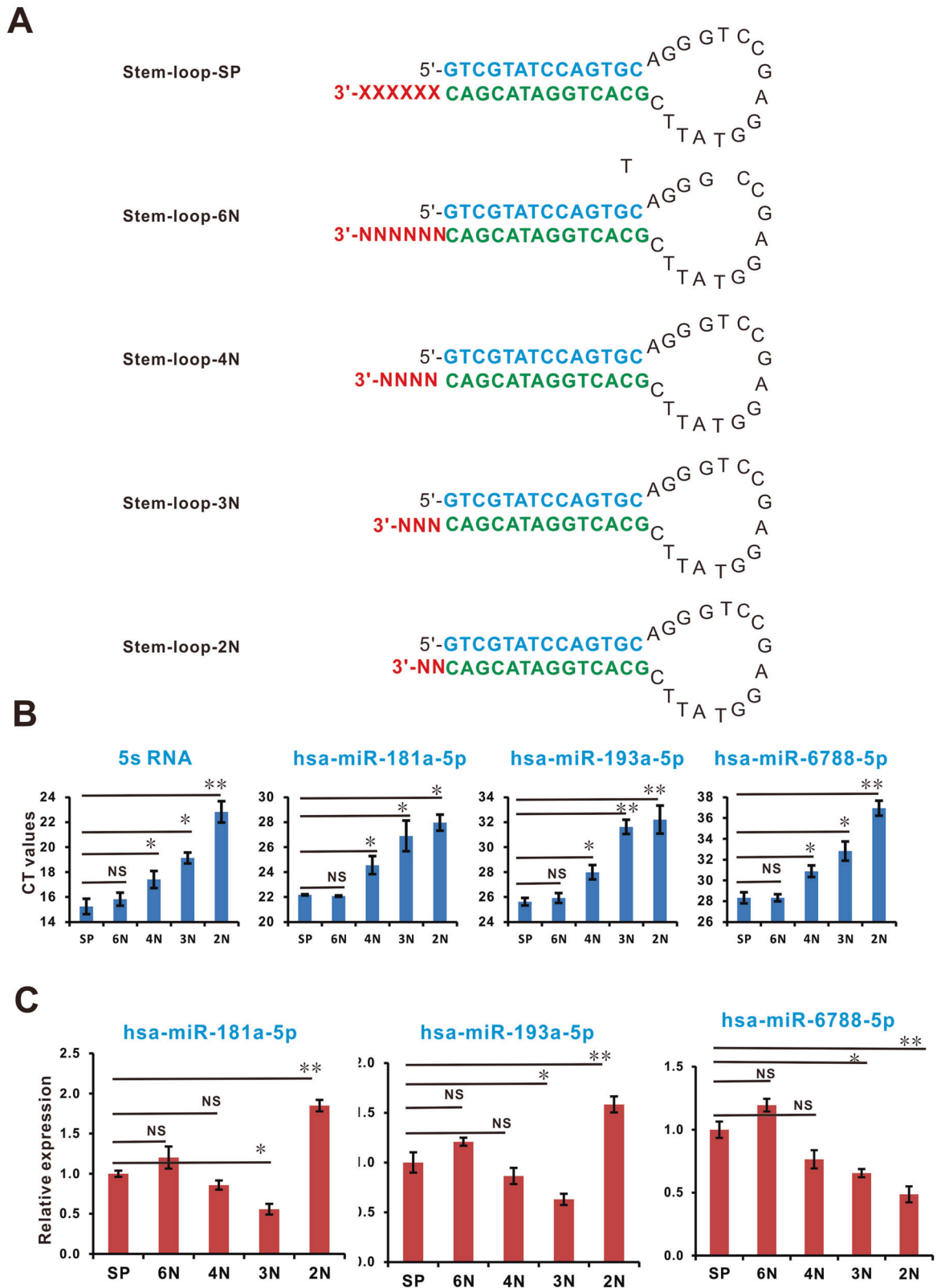


**Fig. 1 Long RNA transcripts ( $\geq 200$  nt) are completely depleted by size selection magnetic beads (SSMBs).** **A** A schematic representation of the long RNA transcript ( $\geq 200$  nt) depletion from total RNA using SSMBs. The extracted total RNA was mixed with the Mag-Bind SSMBs at a volume ratio of 1:1 (v/v, RNA: Beads) for 10 min at room temperature (a). The mixture was subjected to magnetic separation (b) and the short transcripts ( $< 200$  nt) in the supernatant were saved (c), while the long transcripts on the beads were discarded (d). The saved short transcripts ( $< 200$  nt) in the supernatant were purified using the phenol/chloroform method, and can then be stored at  $-80$  °C for reverse transcription reactions (e). The long transcripts ( $\geq 200$  nt) are discarded or saved (f). **B**, **C** The efficiency of long transcript removal with the Mag-Bind SSMBs at different RNA:Beads volume ratios (5:4, 1:1, 1:2, 1:3) was evaluated with an Agilent 2100 Bioanalyzer. A representative gel image (**B**) and the electropherograms (**C**) are shown. One tenth of total RNA without long transcript removal was used as an input control. **D** Randomly selected microRNAs from the SJS1 cells with/without long transcript removal were detected by the TSLP method to evaluate the loss of microRNA after long transcript removal and following small transcript phenol/chloroform purification. NS non-significant.

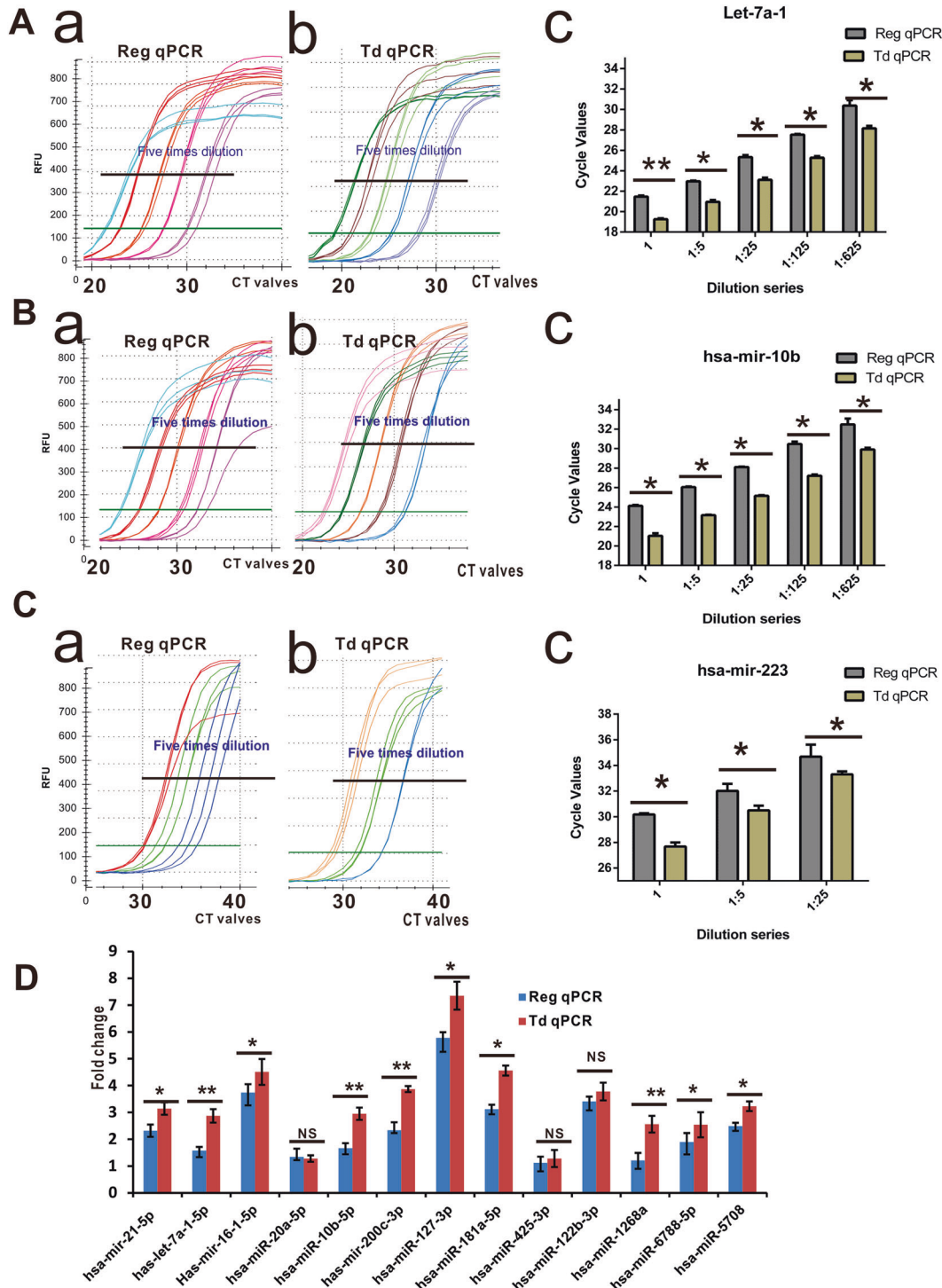
#### LTMT is a simple and convenient method for microRNA profile detection

After optimizing these key steps for microRNA qPCR, we developed a microRNA detection method named LTMT-qPCR (Fig. 4A, left), which combines long transcript removal, a stem-loop-6N universal primer for reverse transcription, and Td qPCR. We compared LTMT-qPCR with the TSLP (Fig. 4A, middle) and USLP methods (Fig. 4A, right). The details of these three microRNA

detection methods are summarized in Fig. 4A, B. We compared these three methods side-by-side using the same starting RNA and same cDNA dilutions. We graphed the  $C_T$  values of 5S RNA, hsa-mir-181a-5p, hsa-mir-193a-5p, and hsa-mir-6788-5p to assess the sensitivity of the three methods. The  $C_T$  values for LTMT and TSLP were equivalent, and both were lower than those for USLP, indicating that USLP has lower sensitivity due to the lack of long transcript RNA removal (Fig. 4C).



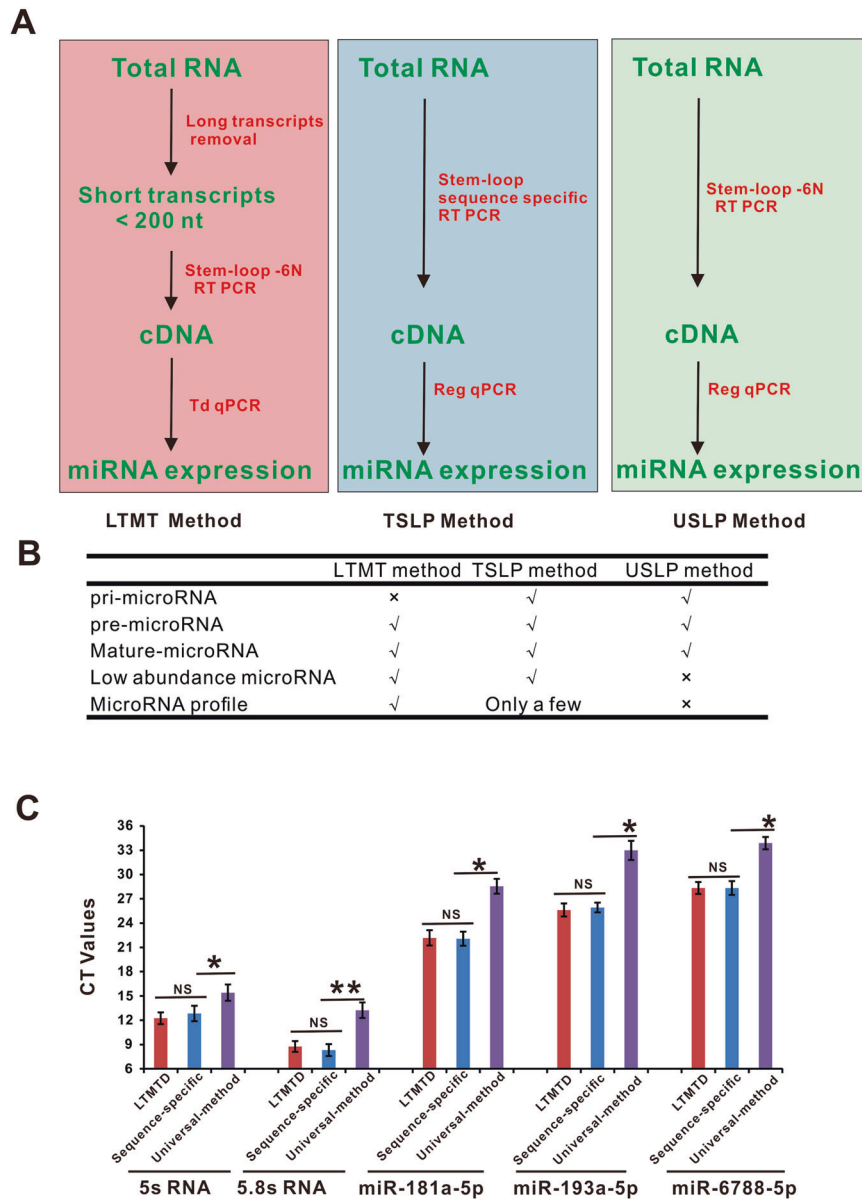
**Fig. 2** The number of randomized deoxyribonucleotides at the 3' end of the USLP influences the efficiency of the reverse transcription reaction. **A** The inclusion of different numbers of randomized deoxyribonucleotides at the 3' end of the USLP. Stem-loop-SP means the last six deoxyribonucleotides at the end of the stem-loop primer are specific to the microRNA. Stem-loop-2N, stem-loop-3N, stem-loop-4N, and stem-loop-6N mean that 2, 3, 4, and 6 randomized deoxyribonucleotides are included at the end of the USLP, respectively. **B** The efficiency of the reverse transcription reaction with different reverse transcription primers was assessed by qPCR. The cycle threshold ( $C_T$ ) values for 5S RNA, hsa-miR-181a-5p, hsa-miR-193a-5p, and hsa-miR-6788-5p were used as representative markers for different abundances of small RNAs. **C** The relative expression of these microRNAs was normalized to 5S RNA. All qPCR reactions were done in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , NS non-significant.



**Fig. 3 A comparison between Reg PCR and Td qPCR in the quantitative amplification of microRNAs with distinct abundance.** A–C A comparison of the amplification efficiency between Reg qPCR and Td qPCR in the quantitative amplification of a high-abundance microRNA (A, let-7a-1), a moderately abundant microRNA (B, hsa-mir-10b) and a low-abundance microRNA (C, hsa-mir-223). SYBR green fluorescence in a dilution series for Reg qPCR (a) and Td qPCR (b). Comparison of the  $C_T$  values between Reg qPCR and Td qPCR reactions (c). **D** Silencing Dicer allowed the differentially expressed microRNAs in SJS1 cells to be more efficiently detected by Td qPCR. Total RNA was extracted and TSLP was used to perform the reverse transcription. The resulting cDNA was diluted 1:25 for Reg qPCR and Td qPCR. The fold changes in microRNA expression were analyzed and graphed. The qPCR reactions were done in triplicate. The numeric values indicate the fold changes compared to the control group. \* $P < 0.05$ , \*\* $P < 0.01$ , NS non-significant.

**LTMT has better detection efficiency than USLP in microRNA profile screening**  
 Nutlin3A is a small molecule antagonist targeting MDM2 and induces cell apoptosis by blocking MDM2-p53 interaction in the

p53 wild-type tumor cell lines such as SJS1<sup>41</sup>. When SJS1 cells were treated with Nutlin3A, the p53 signaling pathway is activated and cell apoptosis is induced. In this process, many p53 signaling pathway-related microRNAs will be upregulated or



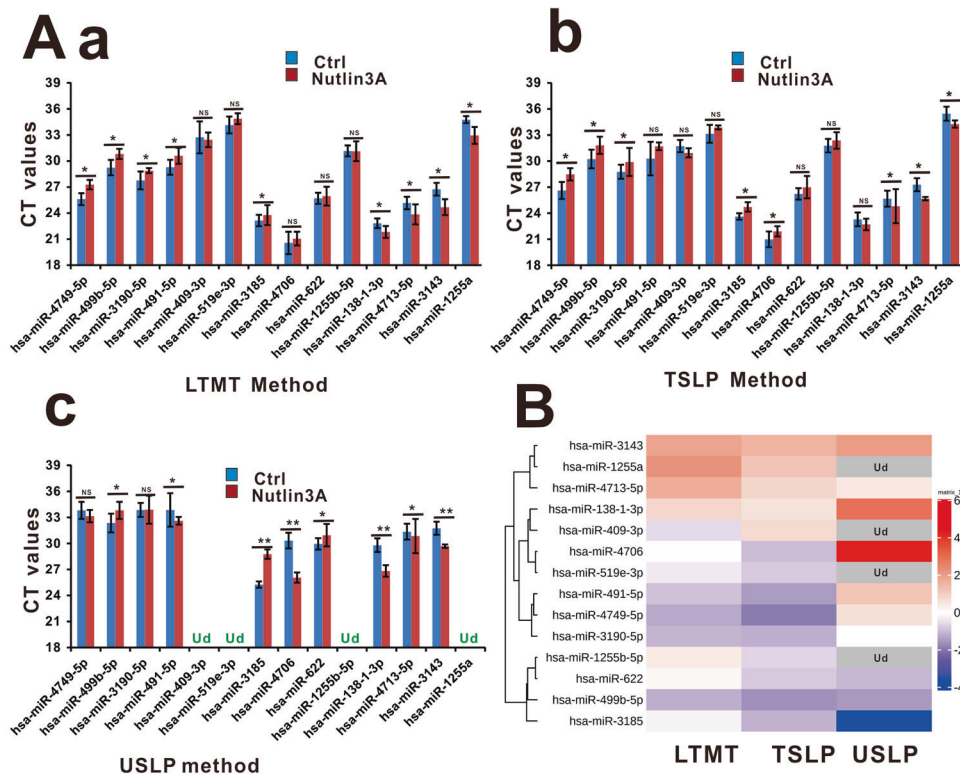
**Fig. 4** LTMT is a simple and convenient method for microRNA profile detection. **A** A schematic representation of the different methods used for microRNA profile detection (left: LTMT; middle: TSLP; right: USLP). **B** A comparison of the three methods. **C** The efficiency of the microRNA profile detection by three methods. The cycle threshold ( $C_T$ ) values for 5S RNA, hsa-mir-181a-5p, hsa-mir-193a-5p, and hsa-mir-6788-5p were graphed to assess the efficiency of detecting different abundances of small RNAs. \* $P < 0.05$ , \*\* $P < 0.01$ , NS non-significant.

downregulated. To further compare the methods and demonstrate their applicability for expression profiling, we treated SJS1 cells with DMSO (control) or 1  $\mu\text{mol}$  nutlin3A for 36 h. We extracted the total RNA and aliquoted them into three parts for p53-related microRNA profile detection using the LTMT, TSLP, or USLP methods. The  $C_T$  values of the selected p53-related microRNAs were analyzed and graphed (Fig. 5Aa–c). The log (fold change) of microRNA expression was used to make a heatmap (Fig. 5B). TSLP is a reliable method for single microRNA detection, but inconvenient for microRNA expression profiling. The expression panel detected with LTMT was similar to that generated from TSLP. However, USLP was not as effective as LTMT and TSLP, especially for low-abundance miRNAs (such as hsa-mir-409-3p, hsa-mir-519e-3p, hsa-mir-1255b-5p, and hsa-mir-1255a) (Fig. 5B). Meanwhile, although the high-abundance microRNAs (hsa-mir-3185, has-mir-4706, and has-mir138-1-3p) were detected with higher fold change in the USLP method, their

$C_T$  was much higher than LTMT and TSLP (Fig. 5B), which makes it less reliable. We think the main reason may be that without long transcript removal, the stem-loop-6N primers have a lower chance to catch the miRNAs, as most of the primers matched to the long transcripts. This leads to the waste of primers and reverse-transcriptase, reducing the reverse transcription efficiency. All of these suggest that LTMT has better detection efficiency than USLP in microRNA profile screening.

#### LTMT represents a simple, inexpensive, and effective method for microRNA profiling

MicroRNA expression profiling is a common and necessary procedure for microRNA-related research. At present, the majority of labs utilize TSLP for the reverse transcription reaction in order to enhance the specificity of detection. However, there are several problems associated with this method. First, total RNA contains large amounts of primary microRNA. Although TSLP can



**Fig. 5** Comparison of the sensitivity and extent of detection for small RNAs by the three different methods. **A** SJS1 cells were treated with DMSO or 1  $\mu$ mol nutlin3A for 36 h, and then the total RNA was extracted for microRNA profile detection with the LTMT method, TSLP method, and USLP method. The cycle threshold ( $C_T$ ) values of the selected microRNAs were analyzed and graphed in (a), (b), and (c). The  $C_T$  values for LTMT and TSLP were similar, while USLP had higher  $C_T$  values. **B** Heatmaps of the microRNA profiles detected with three indicated methods. The microRNA expression panel for LTMT was similar to that of TSLP. The values shown on the heatmap are the logarithmic values of the fold change in expression. Ud under the limit of detection ( $C_T$  values above 39).

specifically capture the mature microRNAs, it also anneals with the primary microRNA, and therefore affects the accuracy of detection. Second, for microRNA profile detection, every microRNA requires a specific stem-loop reverse primer. This is expensive, and the reverse transcription reaction must be carried out many times. To overcome these drawbacks, Yang et al.<sup>35</sup> developed a USLP with eight random nucleotides instead of a specific sequence at the 3' end. Compared with TSLP, USLP saves 75% of the primer cost and 60% of the testing time. However, without the removal of long RNA transcripts, the eight randomized nucleotides will also anneal to the primary microRNA and other long RNAs present. This can lead to the waste of most of the oligonucleotides, as the long RNAs compete with the microRNAs.

To address these problems, we developed the LTMT and optimized it for microRNA profile detection. This method takes advantages of size selection using SSMBs. Due to the great size differences between microRNAs (~22 bp) and long transcript RNA ( $\geq 200$  bp), SSMBs can efficiently and robustly remove long RNA transcripts. After the removal of long RNA transcripts, a USLP with six random nucleotides at the 3' end can efficiently reverse transcribe all of the small RNAs simultaneously. Finally, Td qPCR protocol was incorporated to increase the sensitivity of microRNA detection. In a proof-of-principle study, the microRNA profile detected using the LTMT method was similar to that detected using the TSLP method, but in a more convenient and economical way. While the USLP method is also more convenient than the TSLP method, it was unable to reliably detect several less abundant microRNAs ( $C_T$  values > 39). Thus, LTMT appears to represent the optimal method in terms of sensitivity and efficiency.

It is worth noting that LTMT still has some shortcomings. First, like other methods, LTMT utilizes SYBR green during qPCR, and it

cannot be used to differentiate the expression levels of isomicroRNAs, whose sequences are similar. Second, when the long RNA transcripts are removed during ethanol precipitation, special attention should be paid to avoid losing any short RNAs. Because the short RNA sequenced can be concentrated, users can start with more total RNA in the initial purification step. LTMT removes long transcripts, and therefore can exclude the interference of primary microRNAs, but the precursor microRNAs cannot be excluded.

In conclusion, our newly developed LTMT method is a simple, sensitive, and reliable approach that is suitable for microRNA profile detection.

#### DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author (Z. Z.) upon reasonable request.

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## ACKNOWLEDGEMENTS

We would like to thank Dr Changchuan Niu from the Department of Laboratory Diagnostic Medicine, Chongqing General Hospital, for his technical support.

## AUTHOR CONTRIBUTIONS

X.W. contributed to writing the original draft, and to the investigation. Y.F. contributed to data collection and provided software. X.Y. and L.Z. contributed to the formal analysis and method development. S.Z. and X.K. contributed resources. Y.L. and Q.P. contributed to the validation of the method. Z.Z. contributed to the conceptualization of the study, funding acquisition, project administration, supervision, and reviewing and editing the manuscript.

## FUNDING INFORMATION

The reported work was supported by the China Postdoctoral Science Foundation (2019M663446 to Z. Z.), and the Postdoctoral Program of the Natural Science Foundation of Chongqing, China (cstc2019cyj-bsh0006 to Z. Z.).

## COMPETING INTERESTS

The authors declare no competing interests.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors declare that they have complied with all requirements and guidelines regarding the ethics of the study.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41374-021-00648-9>.

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