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A cost effective MGB free stem-loop qPCR strategy for high precision microRNA profiling

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Abstract

Numerous techniques are already accessible to measure the expression levels of particular microRNAs in biological specimens. One of the most popular methods and, according to experts in this field, the most accurate method available is the Stem Loop method. In this method, the cDNA of each microRNA is first synthesized explicitly with an adapter's help. Finally, the measurement uses general reverse primers, specific forward primers, and a specific probe. One of the problems with this method is that the probe binding site is such that in order to have the appropriate melting temperature (T_m), in most cases, it is necessary to bind the MGB molecule to the probe. This protocol requires much expense and significantly increases the cost of the test. In our innovative method, the adapter and the experiment are designed so that to synthesize a suitable probe, there is no need to add the MGB molecule to it, and the probe alone will have the appropriate T_m .

Keywords miRNA, Stem Loop method, MGB molecule

Introduction

microRNAs, short noncoding RNAs of 19 to 25 nucleotides in length, serve a regulatory post-transcriptional function in gene expression by suppressing target genes at the mRNA level. This inhibition may result in mRNA breakdown or obstruction of protein translation (Sharafeldin et al. 2025). Primary miRNA is the product of initial miRNA transcription, which is mostly carried out by RNA polymerase II. It is then converted into 60–70 nucleotide precursor miRNAs (pre-miRNAs) by

endonucleases. Exportin-5 transports pre-miRNAs to the cytoplasm via the nuclear pores. Subsequently, Dicer processes them to become mature miRNAs (Meng et al. 2017; Ho et al. 2022). The miRNAs interact with many mRNAs, allowing them to play crucial regulatory roles in various physiological and developmental processes. Specifically, the regulation of gene expression through miRNAs plays a vital role in how cells respond to environmental stresses linked to human diseases like cancer. Indeed, various miRNAs can act as oncogenes or as tumor suppressors. The dysregulation of miRNA expression is intricately linked to cancer initiation, progression, and metastasis (Mehdipour et al. 2023). Due to their high abundance and tissue stability, they may be beneficial in developing non-invasive biomarkers for patients with tumors (Ma et al. 2024).

Various techniques are available for the detection and quantification of miRNA expression. The four most conventional methods include northern blotting, Reverse Transcription quantitative PCR (RT-qPCR), In Situ

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Hybridization (ISH), and microarrays, along with next-generation sequencing (RNA-Seq). Furthermore, methods for detection utilizing PCR-Free signal amplification have also been suggested. Each of these approaches presents its own benefits and drawbacks (Yaylak and Akgül 2022). Quantitative polymerase chain reaction (qPCR) is the “target amplification-based” technique most often utilized when looking for low-level miRNAs. This method is widely regarded as highly sensitive for analyzing differential gene expression (Precazzini et al. 2021). This technique provides elevated sensitivity and specificity. It is appropriate for quantifying smaller miRNA panels and can facilitate absolute quantification. It is also suitable for standard measurements in laboratory environments (Dave et al. 2019).

The unique properties of miRNAs pose significant challenges for their detection. Their small size and sequence homology complicate detection regarding selectivity, making amplification a significant challenge (Zhu et al. 2019). Several detection methods can be hindered by the existence of primary (pri-) and precursor (pre-)miRNAs, as well as the possibility of various sequence isoforms (isomiRs) due to minor changes in upstream processing. Furthermore, changes within the same miRNA family might arise from as little as a single base difference, making it extremely difficult to achieve specificity in methods that aim to differentiate between individual members. With a length of around 22 nucleotides—roughly the

same as a conventional PCR primer—miRNAs constitute around 0.01% of the total RNA normally recovered from a sample. Because of this, it appears that sensitive and specialized means of miRNA detection are required. The wide variety of Tms produced by miRNAs due to their short size makes it difficult to optimize the parallel reactions needed to detect them (Hunt et al. 2015). Various methods have been documented for the identification of miRNAs with RT-qPCR. The poly(A) approach is one of them; it uses a universal oligo(dT) primer for reverse transcription (RT) and involves attaching a poly(A) tail to any kind of RNA, including miRNAs. With this technique, miRNA expression can be evaluated at high throughput. The non-specific reverse transcription, however, makes it less specific (Niu et al. 2015). The poly(A) tailing technique commences with the addition of a series of adenosines to the 3' terminus of all RNAs, including miRNAs, facilitated by poly(A) polymerase. The modified RNAs are subsequently transformed into cDNA by reverse transcription employing a universal primer. This primer possesses a defined structure: two variable nucleotides at the 3' terminus, an oligo(dT) sequence centrally located, and a stuffer region at the 5' terminus. A conventional reverse primer is created based on this universal RT primer for quantitative PCR. Ultimately, each miRNA is amplified utilizing a standard reverse primer and a specific forward primer in a SYBR Green-based quantitative PCR test (Lotfi et al. 2024).

A more targeted method, stem-loop RT-qPCR, was created by Chen et al. (Chen et al. 2005) in 2005. Detecting and amplifying mature miRNAs has become much easier with this new and efficient method. The stem-loop transcription primer has a 3' overhang that matches the target miRNA and is structured in a hairpin configuration, as shown in Fig. 1. A universal reverse primer, a Taq-Man probe, and a miRNA-specific forward primer with a 5' adaptor are used for PCR amplification. For more accurate miRNA identification, this design ensures higher specificity and gives more leeway in primer selection (Cao et al. 2023). This approach involves a portion of the probe being precisely linked to an area of the cDNA associated with the miRNA. At the same time, the other part is connected to the region related to the adaptor (Chen et al. 2005). Adding MGB (minor groove binder) to the Taq-Man probe raises the melting temperature of tiny probes, which is necessary since there is a limited sequence between the PCR primers on the hairpin. Consequently, shorter probes can be used because the MGB probes can form highly stable duplexes with single-stranded DNA targets. In addition to higher melting temperature, they offer more specificity and sensitivity than conventional DNA probes due to improved hybridization characteristics (Schmittgen et al. 2008; Xie et al. 2025).

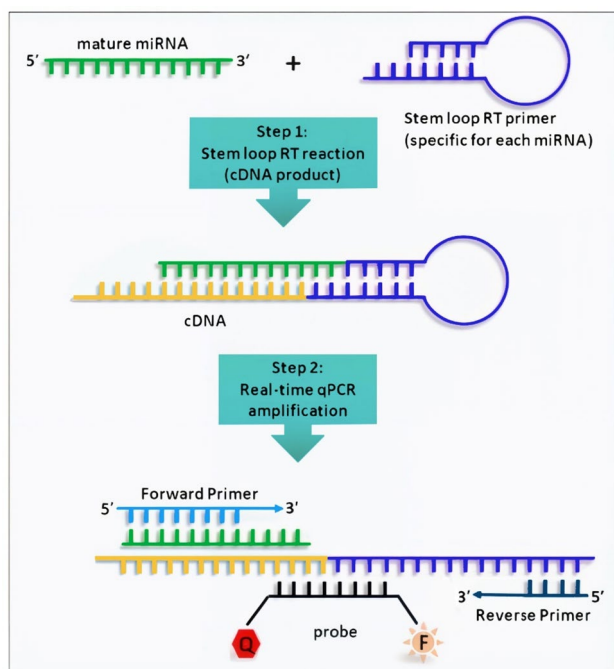


Fig. 1 Reaction steps. In order from top to bottom. The first step is the synthesis of specific cDNA from miRNA. The second step is the detection of miRNA using RT-qPCR method by binding a forward primer, reverse primer, and a probe

MGB probes are costly, so we aim to create an innovative, specialized, and economical approach to measuring miRNA expression. We have termed this method “MGB-Free RT-qPCR” since it avoids using MGB in RT-qPCR by using specific probes with suitable Tms. We aim to use bioinformatics analysis to restructure the adaptor. This means MGB does not need to be coupled to the probe during RT-qPCR. As such, this change greatly lowers the test's cost.

Materials and methods

Bioinformatics analysis and synthetic oligonucleotides

The miRNA sequences were retrieved from the miR-Base database. Compared to the Chen method, the probe binding site on the adaptor was extended. Next, the adaptor structure was analyzed using the UNAFold web server. The sequences of the primers (forward and universal reverse), adaptors, and miRNAs are provided in Table 1. Metabion GmbH synthesized the oligonucleotides.

cDNA synthesis

Whole blood samples were collected, and their plasma part was divided. After the samples were gathered, an RNX-PLUS (Sinaclon, IRAN) reagent was added. According to the manufacturer's protocol, the RNA was extracted using an RNX-PLUS solution. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) was used to evaluate RNA quantity and purity. 50 ng of extracted miRNA was used for cDNA synthesis. cDNA synthesis was performed using a first-strand synthesis kit (Takara Bio Inc.) in a volume of 10 µl. The reaction mixture contained 2 µl of 5x RT buffer, 0.5 µl of ExcelRT™ Reverse Transcriptase (200 U/µl), 0.5 µl RNase Inhibitor (20 U/µl), 0.5 µl dNTP Mix (10 mM each), and 4.5 µl of total RNA, with the final volume adjusted to 10 µl using DEPC-treated water. The RT reaction in the peqSTAR 96X thermocycler (PEQLAB Biotechnologie GmbH, Germany) was performed with a temperature profile of 16 °C for 30 min, then 42 °C for 30 min, followed by 85 °C for 5 min for inactivation.

Quantitative PCR

qPCR reactions were performed in a final volume of 10 µl using SYBR Green PCR Master Mix (Takara Clontech), containing 1.5 µM of forward primer, 0.7 µM of reverse primer, and 1 µl of cDNA template. All reactions were conducted in duplicate. Amplification was carried out on a 72-well Rotor-Gene Q real-time PCR system under the following cycling conditions: an initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. These clearly defined reaction components and cycling parameters were used to ensure experimental reproducibility. The results obtained using this protocol were subsequently compared with those reported by Chen.

A probe-based test was done in the next step. A unique forward primer, a universal reverse primer that anneals to the 5' adaptor sequence, and a unique probe for every miRNA target were used in this approach. The overall reaction volume was 10 µl comprising the Taq man probe Master Mix (Takara Clontech), 1.5 µl forward primer, 0.7 µl reverse primer, 0.2 µl target-specific probe, and 1 µl cDNA product. Reactions were done in duplicates and incubated in a 72-well Rotor Real-Time Detection System (Rotor-Gene Q) at 95 °C for 15 min, then 45 cycles of 95 °C for 15 s and 60 °C for 1 min. We compared the findings of our approach to those of Chen.

MiRNA profiling in plasma samples from patients with colorectal cancer

All processes using plasma samples from patients with colorectal cancer and specimens were acquired from healthy individuals who had not been diagnosed with colorectal cancer or any other conditions.

The cDNA synthesis was performed using a specifically designed adapter sequence. Subsequently, 1 µl of the synthesized cDNA was used for quantitative analysis of miR-31 in samples from patients with colorectal cancer, and miR-31, miR-20, miR-18, and miR-378 were analyzed in plasma samples obtained from healthy people. The results are shown in Fig. 2.

Repeatability

The repeatability of the MGB-Free method was assessed against the other methods by measuring imprecision, expressed as the standard deviation of Ct values obtained from triplicate measurements. (Table 2). In this study, we compared the Ct values obtained from different miRNA quantification methods, including Chen's method utilizing a probe, Chen's method employing SYBR Green, MGB-Free method with a probe, MGB-Free method with SYBR Green, and the Poly(A) method. The expression levels of the synthesized microRNAs miR-18, miR-20, miR-31, and miR-378 were measured, and the obtained results were evaluated by the *CLSI EP 15-A3* guidelines.

Table 1 Primer and probe information used in this study

miRNA	Primer/Probe	Sequence (5'–3')	Length (nt)
hsa-miR-31-5p	Forward primer	AAGCGAGGCAAGAT-GCTGG	19
hsa-miR-31-5p	Reverse primer	CGTGGTTAGGGTCCGAG-GTA	20
hsa-miR-31-5p	Probe	FAM-CCCACGTGGAGGAC-GACGAGCTATG-BHQ1	25

Forward primer (F), Reverse primer (R), and specific probe (P) for hsa-miR-31-5p. The red region on the adaptor indicates the binding site for the reverse primer, and the green region indicates the specific binding site of the adaptor to the miRNA. The specific binding site of the probe on the adaptor is underlined. The specific sequences of the forward, reverse, and probe for hsa-miR-31-5p are provided

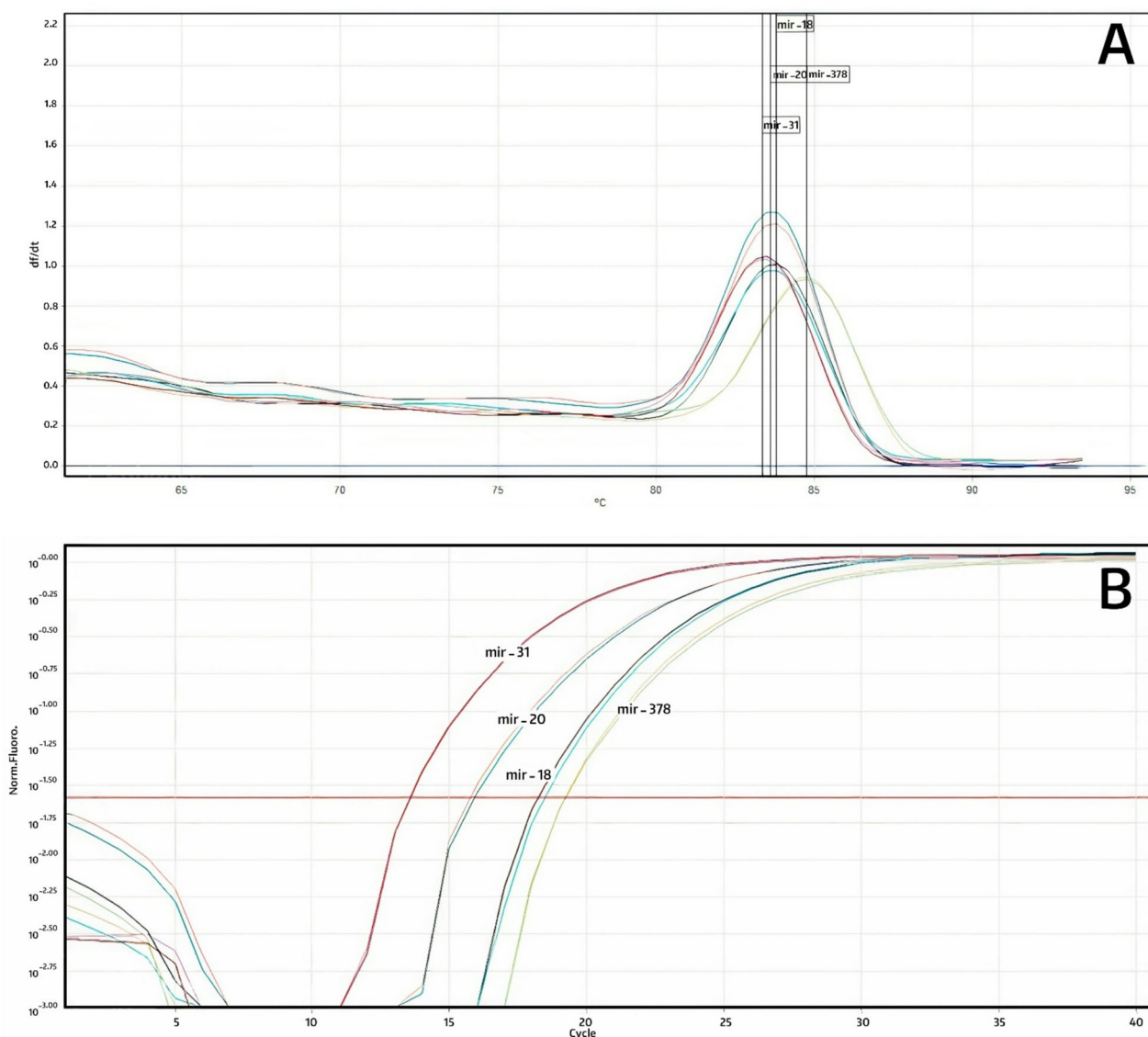


Fig. 2 **A** Melting Curve and **B** Amplification Curve for miR-31, miR-20, miR-18 and miR-378

Limit of detection (LoD) and dynamic range

Limit of detection (LoD), refers to the lowest amount of the target analyte in a sample that can be detected in at least 95% of cases. To determine the LoD, serial dilutions are prepared from a known analyte concentration sample. To evaluate the LoD of the MGB-Free method, we targeted hsa-miR-31, which was synthetically produced. Ten-fold serial dilutions of hsa-miR-31 were prepared, and each sample was analyzed in twenty replicates. Serial dilutions ranged from approximately 1 to 10⁶ copies per reaction. Each RT reaction received 100 ng of bacterial total RNA to produce a complicated background mimicking biological sample, producing similar outcomes.

The dynamic range for hsa-miR-31 was evaluated to further investigate the adaptability and quantitative performance of the qRT-PCR assay. Synthetic oligonucleotides

were serially diluted across seven orders of magnitude (10 to 10⁷ copies per reaction) and evaluated.

Results

General assay design

According to the MGB-free amplification system in Chen method, reverse transcription is performed using specific primers, each 60 nucleotides long. The six nucleotides at the 3' end of these primers are designed as reverse complements to the six nucleotides at the 3' end of the miRNA sequence, enabling the two regions to bind and allowing cDNA synthesis to occur.

The cDNA is then quantified by RT-qPCR using a specific forward primer, a probe, and a universal reverse primer complementary to a portion of the RT primer. The forward primer is specifically designed based on

Table 2 Average Ct values, standard deviations and coefficient of variation of three miRNAs measured in triplicate using three distinct techniques. Total RNA was extracted from the human plasma sample

	MGB-Free with a probe			MGB-Free with SYBR Green			Chen method with a probe			Chen method with SYBR Green			Poly(A)		
	Ct	St.dev	CV	Ct	St.dev	CV	Ct	St.dev	CV	Ct	St.dev	CV	Ct	St.dev	CV
miR-18	17.5	0.21	1.2%	18.7	0.28	1.5%	17.9	0.18	1.0%	19.3	0.31	1.6%	21	0.43	2.0%
miR-20	16.4	0.20	1.2%	17.6	0.27	1.5%	16.8	0.22	1.3%	18.3	0.32	1.7%	20.2	0.45	2.2%
miR-31	14.5	0.19	1.3%	15.9	0.21	1.6%	14.9	0.15	1.0%	16.5	0.29	1.8%	18	0.41	2.3%
miR-378	18.8	0.31	1.6%	20.3	0.38	1.9%	19.2	0.28	1.5%	20.8	0.4	1.9%	23.1	0.54	2.3%

CT: Cycle Threshold; St.dev: Standard deviation; CV: Coefficient of variation

the 5' end nucleotides of the miRNA, while the probe is designed based on the six nucleotides at the 3' end.

Bioinformatics analyses confirm that the reverse primer and probe have appropriate Tm values, and the RT primer forms a stem-loop structure, as shown in Fig. 3.

The results obtained from the qPCR assay using Syber Green were as follows: the melting curve analysis of the amplified miRNAs revealed single, sharp peaks, confirming the specific amplification of each target miRNA. Additionally, the amplification plots showed that all miRNAs under investigation displayed low and appropriate Ct values.

A comparative analysis of our method against the poly(A) tailing method and stem-loop RT-qPCR methods (both with and without MGB probes) demonstrated that, under identical experimental conditions, the Ct values obtained with our method were similar to those obtained with the stem-loop method employing MGB probes. Notably, the Ct values from our method were significantly lower than other methods without MGB probes. These findings provide strong evidence for the enhanced analytical sensitivity of our developed method.

Repeatability

The performance of the MGB-Free method (with a probe and SYBR Green) was compared to the Chen (utilizing a probe and SYBR Green) and poly(A) methods. While the overall strategy of the MGB-Free and Chen methods is similar, the MGB-Free method incorporates modifications to the RT primer sequence and probe structure, including a more extended sequence and the removal of MGB. In contrast, the poly(A) method utilizes poly(A) polymerase to add a poly(A) tail to the 3' ends of miRNAs, followed by reverse transcription of all miRNAs using oligo(dT) primers. All methods demonstrated high repeatability, as indicated by their low standard deviation values (Table 2).

Limit of detection and dynamic range

The LoD was calculated using specific concentrations of hsa-miR-31 and ten-fold serial dilutions. According to the obtained results, it was equivalent to 10 copies (Table 3).

The results of the Dynamic Range Evaluation Test revealed significant linearity across seven orders of magnitude. The standard curves for hsa-miR-31 revealed consistent amplification efficiency (105%) with slight variation between repetitions (Fig 4). These findings imply that the MGB-Free platform is generally relevant for the sensitive and quantitative detection of many miRNAs, preserving performance across targets and concentrations.

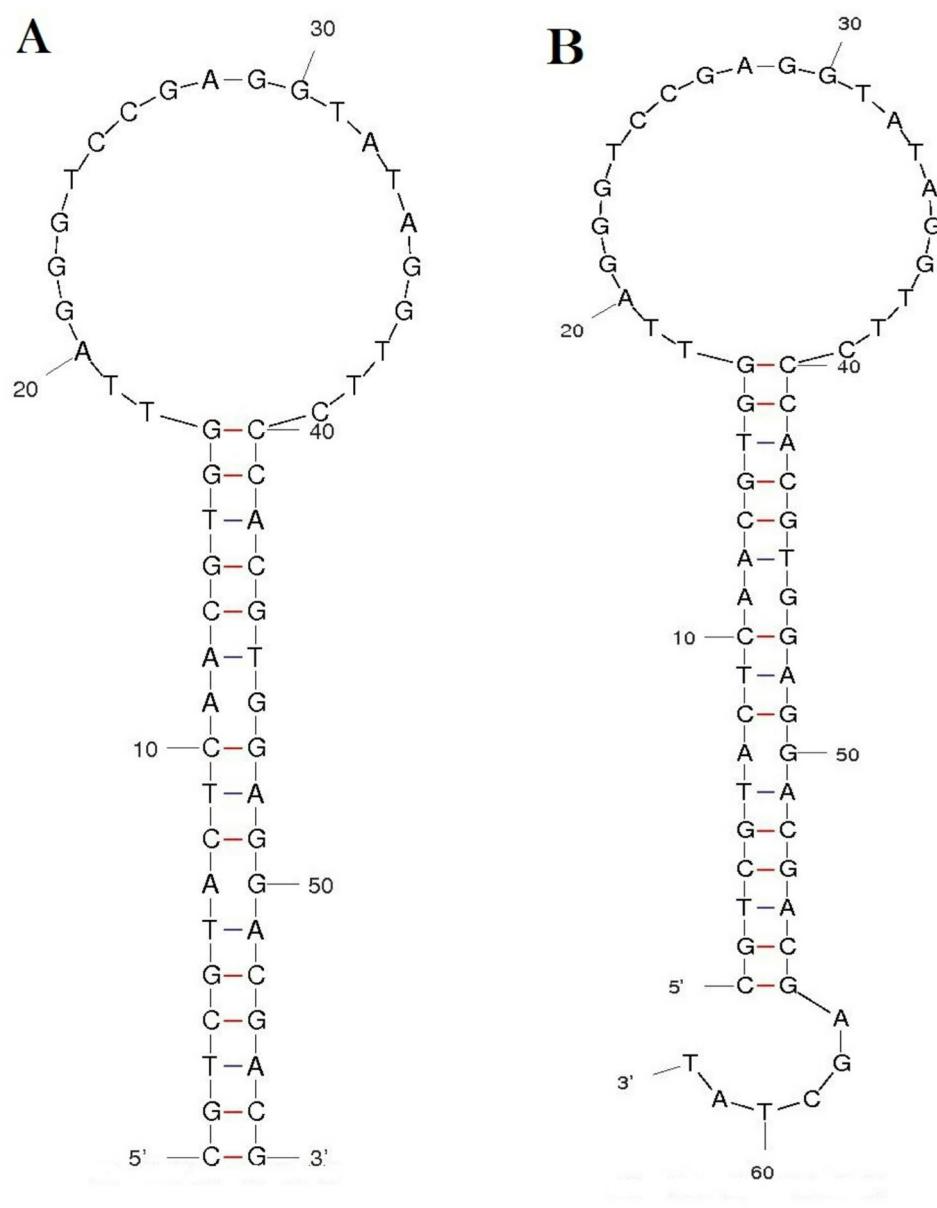


Fig. 3 Analyzing adapter structure bioinformatically using UNAFold software (version 2.3). **A** Stem loop structure of adapter without miRNA-specific binding region. **B** Stem loop structure of the adapter along with 6 nucleotides complementary to the 3' end of hsa-miR-31-5p

Table 3 LoD for MGB-Free method			
Concentration (copies/ RXN)	Total Test	hsa-miR- 31 Positive Detection	Detect- ion Rate
10 ⁶	20	20	100%
10 ⁵	20	20	100%
10 ⁴	20	20	100%
10 ³	20	20	100%
10 ²	20	20	100%
10 ¹	20	20	100%
1	20	12	60%

Multiplexing of the reverse transcription

By synthesizing cDNA in a multiplex manner, we can significantly improve the efficiency of our workflow, conserve valuable reagents, and maximize instrument utilization.

We multiplexed the RT step with the unique adapter sequence and RT-qPCR assays by measuring the expression of miR-31, miR-20, miR-18, and miR-378 expression in plasma samples obtained from healthy people. A total of 150 ng of extracted miRNA was used for cDNA synthesis. Subsequently, 1 µl of the synthesized cDNA was used to analyze miRNA levels using qPCR quantitatively. For qPCR, specific forward primers and a universal

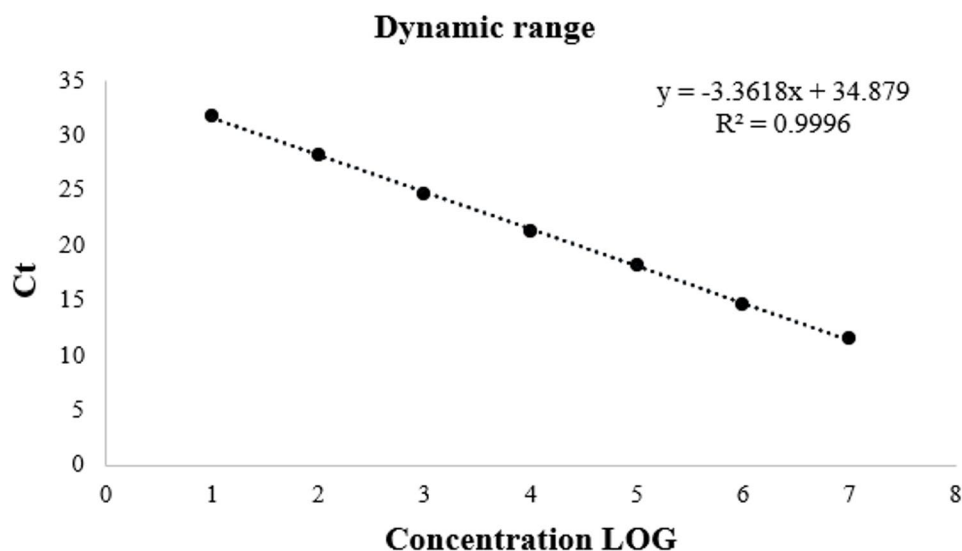


Fig. 4 Dynamic range of MGB-Free method. As shown in the figure, the method has an acceptable linear range in the span of 10 to 10^7 copy numbers of miR-31 ($R^2 = 0.9996$)

reverse primer were used. The melting curves of the miRNAs were analyzed. The melting curves of the miRNAs showed single peaks, indicating their specific amplification. Furthermore, the amplification plots demonstrated that all the examined miRNAs exhibited low and suitable Ct values. The results are shown in Fig. 5.

Discussion

Measuring microRNA expression in biological samples can be achieved through a range of methods, some proprietary and held by a limited number of large companies. While each method presents its strengths and weaknesses, no single approach is universally preferred.

High-throughput techniques such as Microarray and RNA-seq can simultaneously measure the expression of numerous microRNAs; however, they tend to have lower accuracy and sensitivity. In contrast, the more precise and commonly used methods rely on qPCR (Sharafeldin et al. 2025).

Several alternative methods have been developed based on the original stem-loop technique, each offering distinct characteristics. One such variation eliminates using a probe during the qPCR phase, relying exclusively on forward and reverse primers (Ho et al. 2022). In this method, SYBR Green dye is employed to track the amplification of PCR products instead of a probe, which helps lower testing costs. However, this approach has reduced specificity since the only determinant of specificity in qPCR is the forward primer, and it lacks a dedicated probe. Furthermore, using specific probes labeled with various fluorescent dyes allows for the simultaneous analysis of multiple miRNAs in a multiplex format; unfortunately, this method does not have that capability.

Another option utilizes a universal probe. In this approach, the probes attach only to a section of the adapter and do not target any part of the miRNA. As anticipated, this leads to decreased specificity compared to methods where the probe specifically targets the miRNA (Ho et al. 2022; Mehdipour et al. 2023).

The main issue with the probes used in the aforementioned methods is that the binding region of the probe to the adapter is structured and sized to result in a very low T_m for the probe. To compensate for this low T_m , attaching the probe to an MGB molecule becomes necessary, which significantly increases the probe's cost (Ma et al. 2024).

In our innovation protocol, we have redesigned the adapter structure to eliminate the need for MGB attachment during the qPCR stage. This modification dramatically reduces testing costs while preserving sensitivity and specificity, similar to the method proposed by Chen et al., as confirmed by our studies.

The method involves two key stages. In the first stage, miRNAs are converted into cDNA using a specially designed adapter. The adapter sequence includes six specific nucleotides at its 3' end complementary to the miRNA, ensuring specific binding between the adapter and the miRNA. Additionally, the 5' end of the adapter contains a region for binding a universal reverse primer. The probe binding site is also located at the 3' end of the adapter, allowing it to simultaneously bind 6 to 7 nucleotides from the miRNA's 3' end and approximately 17 to 20 from the adapter, depending on the assay type. This design maintains the stem-loop structure of the adapter while achieving an optimal T_m for the probe without requiring MGB, which is a key factor in significantly

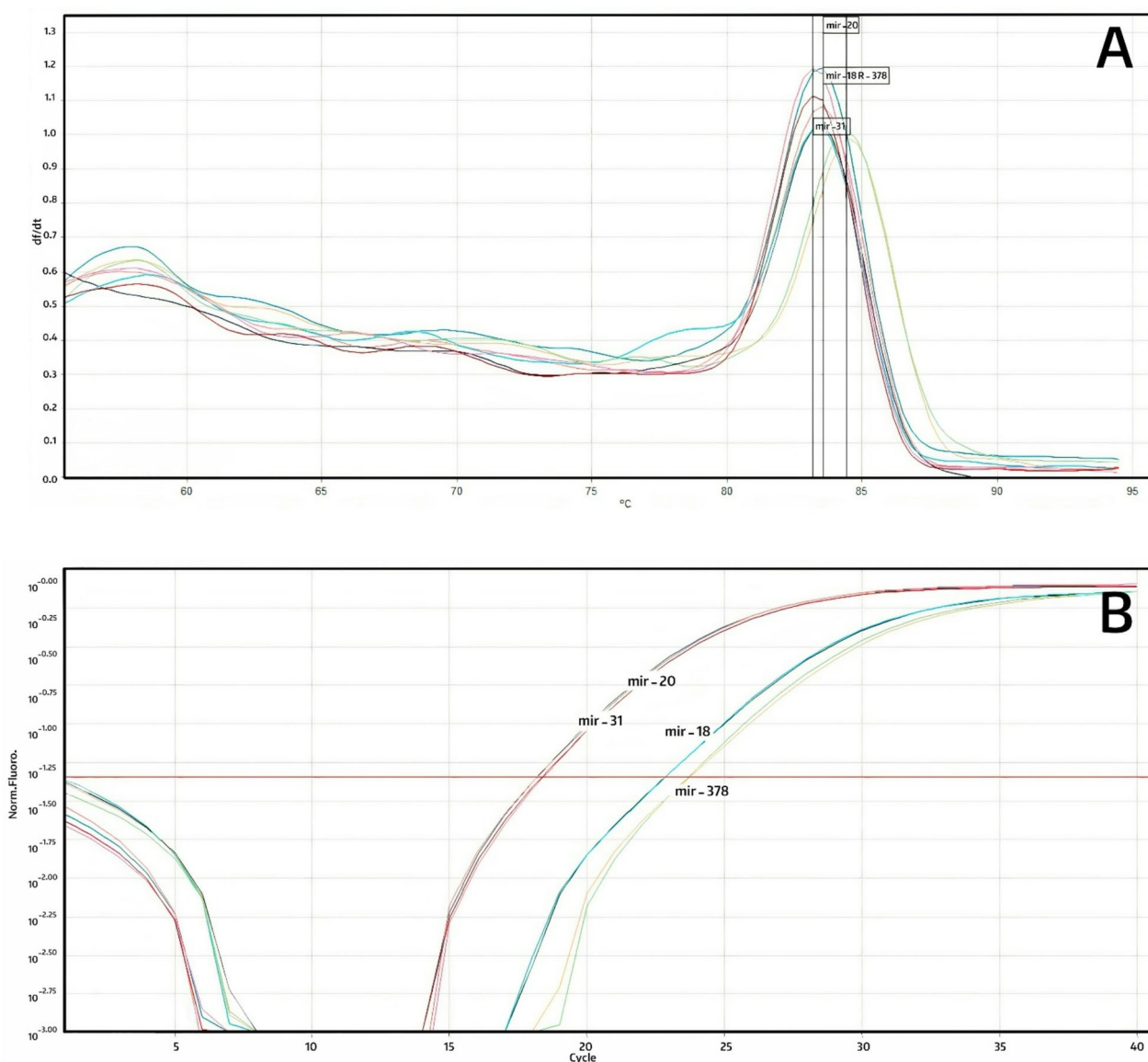


Fig. 5 Multiplex cDNA synthesis reaction and then qPCR reaction for miR-31, miR-20, miR-18 and miR-378 on the plasma samples of a healthy person. **A** Melting Curve and **B** Amplification Curve

lowering testing costs and making the method far more cost-effective.

In the second stage, qPCR is performed on cDNA samples. During this step, a universal reverse primer that binds to the 5' end of the adapter is used alongside a specific forward primer and a specific probe for each miRNA. This method demonstrates exceptional specificity because both the forward primer and probe are tailored to individual miRNAs. Furthermore, since each probe is unique to its corresponding miRNA, multiplex assays can be performed by using different fluorescent labels for each miRNA.

Conclusion

The innovative method presented here, featuring a uniquely designed adapter and reaction components, generates probes with optimal melting temperatures (T_m) without requiring MGB conjugation. This advancement significantly reduces assay costs and streamlines and accelerates the workflow. Moreover, the platform readily adapts into a comprehensive kit format for precise quantitative and qualitative assessment of various miRNAs in biological samples. Beyond research applications, this approach holds substantial promise for integration into clinical diagnostic kits based on miRNA measurement, offering a cost-effective, user-friendly solution for molecular diagnostics and translational research.

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Author contributions

E.L, F.K and M.A performed the experiments. A.G and F.G analyzed the data and interpreted the results. M.B conceived and designed the study. E.L, F.K and M.A prepared the manuscript. M.B, and E.L reviewed during the manuscript preparation and revised the manuscript.

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Data availability

The data that support this study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate

It was carried out in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of the Affiliated Hospital of Iran University of Medical Science (IR.IUMS.REC.1397.1217). All patients and healthy volunteers gave their informed consent to use their blood samples and data.

Competing interests

The authors declare that they have no potential conflicts of interest regarding research, authorship, or publication in this article.

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