# Analytical challenges and technical limitations in assessing circulating MiRNAs

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**Summary**
MiRNAs are emerging as promising biomarkers in cardiovascular diseases and may constitute a novel mechanism of intercellular communication. Accurate quantification of circulating miRNAs is essential. A variety of technological approaches and platforms have been developed with increased sensitivity and specificity for the detection and quantification of circulating miRNAs. In this review, we focus on the technical aspects and discuss the analytical challenges in profiling circulating miRNAs.

**Keywords**
microRNA, biomarker, cardiovascular, diabetes

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## Introduction

MicroRNAs are small (20~23 nucleotides) noncoding RNAs that act as posttranscriptional regulators of gene expression. It is estimated that the human genome encodes more than 1,000 miRNAs. Most mammalian miRNAs are transcribed by RNA polymerase II into a 5'-capped, spliced and polyadenylated primary miRNA transcript, the pri-miRNA (1). The pri-miRNAs are processed in the nucleus by the endonuclease Drosha into 70–100 nucleotides long hairpin-shaped precursors (pre-miRNA). Pre-miRNAs are exported from the nucleus to the cytoplasm where they are cleaved by the endonuclease Dicer to generate a double stranded miRNA. This imperfect miRNA-miRNA* duplex is then loaded into a multicomponent protein complex, the RNA-induced silencing complex (RISC) by associating with Argonaute proteins (Ago) (2, 3). The mature miRNA is incorporated into the complex that recognises specific targets and induces posttranscriptional gene silencing by either translational repression or mRNA degradation through deadenylation (4, 5). Thereby, this conserved class of elements can modulate diverse biological processes (6).

MiRNAs are present in body fluids including plasma and serum. Numerous studies explored the potential of miRNAs as novel biomarkers of cardiovascular disease (7). Extracellular miRNAs show remarkable stability and resistance to degradation (8). Initially, this was explained by the containment of miRNAs in microvesicles (microparticles, exosomes, apoptotic bodies) that physically shield miRNAs from endogenous RNAse activity (9–11). Later studies also revealed the presence of miRNAs in protein complexes (nucleophosmin, Ago2) and lipoproteins (low-density lipoprotein [LDL], high-density lipoprotein [HDL]). Thus, alternative mechanisms exist for miRNA secretion and transport in the circulation (12–14).

## I. Sample collection and handling

MiRNA profiling can be performed in plasma and serum, but preanalytical variation can hamper their quantification. Effective removal of cellular components is essential. Both plasma (supernatant of blood collected in the presence of an anticoagulant) and serum (supernatant of coagulated blood depleted of fibrinogen) can be contaminated with cell remnants from erythrocytes, leukocytes and platelets. Erythrocytes have high levels of miR-16 and miR-451, which correlate to the degree of haemolysis in plasma samples (15–17). In serum, the concentration of platelet microparticles is more than 10-fold greater than that observed for plasma (65 μg/ml vs. 4.4 μg/ml, respectively) (18). The centrifugation protocol will also influence the miRNA content (platelet-rich vs. platelet-poor plasma). For plasma, the anticoagulant is important. Heparin has a dose-dependent inhibitory effect on PCR quantification. Unlike EDTA, it cannot be removed during RNA extraction and binds to calcium and magnesium in the PCR Mastermix (19, 20). In contrast to heparin that renders plasma samples unsuitable for miRNA assessment, citrated plasma samples can be used for circulating miRNA quantification. This anticoagulant, however, is also not ideal as it causes dilution of plasma and may trigger haemolysis (21). Therefore EDTA is the anticoagulant of choice for miRNA quantification. Frozen plasma and serum samples are typically stored at –80°C. Although previous studies suggested that

extracellular miRNAs are resistant to RNase degradation and repeated cycles of freeze-thawing do not affect their integrity, any unnecessary freeze-thawing should be avoided since only a handful of highly abundant miRNA targets were quantified in these studies (8, 17, 22).

## II. RNA extraction

Low yields of RNA further hamper miRNA quantification in plasma and serum. The relatively high protein and lipid content of these body fluids can interfere with RNA isolation. Early protocols were based on the use of TRIzol reagent, a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilises biological material and denatures proteins. After solubilisation, the addition of chloroform results in phase separation with the RNA remaining in the aqueous phase. RNA extraction was completed by isopropanol precipitation and followed by resuspension of the RNA pellet. Although TRIzol extraction was an effective method for isolating small RNAs, the frequent phenol contamination in the RNA preparations and RNA pellets that were difficult to resuspend rendered this protocol problematic (23). Modifications, including a combination of phenol/ guanidine-based sample lysis with routine glass-fiber binding purification of RNA, led to the development of commercially available kits that facilitated lysis and removal of proteins and lipids and elution of high quality RNA (24, 25). The presence of a carrier, such as glycogen and bacteriophage MS2, enhanced the efficiency of the RNA extraction (26, 27). Due to the low yield of RNA from body fluids, a fixed volume of eluted RNA is routinely used for all downstream applications (8, 24).

## III. Detection of circulating miRNAs

There are several challenges regarding the analysis of circulating miRNAs: 1) their short length, 2) the unequal melting temperatures, 3) the existence of miRNAs that often display only single nucleotide differences, and 4) their low abundance. High-throughput sequencing technologies offer a unique opportunity for detecting and profiling miRNA pools with unprecedented sensitivity (28) and can be applied to circulating miRNAs. However, this is only feasible for a limited number of samples due to high costs (29). Also, 2–3 ml of plasma/ serum samples must be processed in order to obtain sufficient input RNA. For most laboratories, quantitative real-time PCR (qPCR) is still the gold standard for assessing circulating miRNAs in large clinical studies with its superior sensitivity compared to northern blotting, cloning, primer extension, and microarrays (30–35).

## IV. Profiling of circulating miRNAs

Arrays can be used to monitor the expression levels of numerous miRNAs. Although considered less sensitive than qPCR quantification, they are essential tools for the discovery phase. A wide range of technical platforms was developed for miRNA screening, including microarrays, bead-based detection, amplification-based technologies and small RNA cloning. Features unique to miRNAs such their small size, low abundance (they represent only ~0.01% of total cellular RNA), the existence of closely related miRNA families and the need to eliminate array signal from pre-miRNAs and pri-miRNAs led to profound modifications of the existing platforms and the development of novel profiling solutions (36–40). Here, we will discuss the technologies that utilise minute amounts of input RNA, but are suitable for the detection of less abundant miRNAs and thus have been employed in global expression profiling of circulating miRNAs (▶Table 1).

### a) Oligonucleotide microarrays

Microarray-based miRNA profiling is a promising high-throughput technique for assessing miRNA expression levels. However, the probe design for miRNA microarrays is critical. The capture probes, synthetic oligonucleotides or cDNA fragments that display high specificity and affinity for individual transcripts, have to be adapted to facilitate binding to miRNAs. Due to the short length of miRNAs, probes melting temperatures ($T_m$) may vary between 45°C and 74°C. Thus, a single hybridisation temperature is suboptimal for most miRNA targets. At a medium hybridisation temperature, capture probes with lower $T_m$ values will yield lower signals, while capture probes with higher $T_m$ values will display impaired nucleotide discrimination and lower specificity (39). To overcome this hurdle, adjustments of the probe length were performed. The probes were selected based on their sequence and size and displayed high specificity of detection for closely related mature miRNAs. The enzymatic labelling had little bias as it included attachment of a single fluorophore-labelled nucleotide to the 3' end of each miRNA with high yield and minimal samples manipulation. Hybridisation to the microarray was carried out under conditions that resulted in near-equilibrium binding and high hybridisation yields for most miRNAs (39). This platform was shown to be extremely accurate although some variability can be observed at low concentrations (30, 41). No fractionation or amplification is required and 100ng of input total RNA has been successfully used to assess the miRNA profile in plasma samples (42).

### b) LNA miRNA expression profiling

Locked nucleic acids (LNA) are nucleotide analogs that are constrained in the ideal conformation for Watson-Crick binding and enable more rapid and stable pairing with the complementary nu-

**Table 1: Comparison of the profiling platforms applied to circulating miRNA screening.**

| Method | Principle | Strengths | Weaknesses | References |
|---|---|---|---|---|
| Oligonucleotide microarrays | Adjustment of the capture probe length. Probes selected based on sequence and size and display high specificity of detection for closely related mature miRNAs. | High throughput. No fractionation or amplification required. Input total RNA 100 ng. | Depending on the concentration, may lack in precision, with higher variability observed at lower concentrations. | Wang (2007) Sah (2009) Ach (2008) |
| miRCURY LNA microRNA arrays | LNA analogs incorporated into capture probes. All miRNA targets hybridize to the array with equal affinity under high stringency hybridization conditions. | Input total RNA 30 ng. High sensitivity. | Capture probe design may affect the hybridization. | Castoldi (2006, 2008) |
| Bead arrays | Incorporates an enzyme-assisted specificity step, a solid-phase primer extension to distinguish between members of miRNA families. A universal PCR is used to amplify all targets prior to array hybridization. | Higher specificity and sensitivity due to solution phase between probe and target. Input total RNA 100 ng. | Low throughput. Fractionation is essential to enrich for small RNAs. Possible bias. | Chen (2008) |
| Microfluidic primer extension arrays | An elongation step is included using the Klenow fragment of DNA polymerase I. All reactions are performed on the Biochip. | Input total RNA 20 ng. Extremely sensitive and does not require amplification. | Not extensively tested. | Vorwerk (2008) |
| Amplification-based arrays | In effect a qPCR reaction. Both stem-loop primers and LNA primers have been used. | High sensitivity and enhanced specificity compared to microarrays. Input total RNA 20 ng. | Low throughput. Concerns about accuracy due to the preamplification reaction and about specificity due to reported false positives. | Chen (2005) Jensen (2011) |

cleotide strand thereby increasing specificity and detection efficiency for miRNAs. LNA analogs were also incorporated into capture probes. This different strategy of probe design resulted in a major improvement in sensitivity and accuracy. By adjusting the LNA content and the length of the probes, the capture probes have been $T_m$ normalised to ensure that all miRNA targets hybridise to the array with equal affinity under high stringency hybridisation conditions. Hence, miRNA profiling is possible with as little as 30 ng of total RNA (43, 44). LNA miRNA arrays have been applied to screen plasma samples from hypertensive patients (45).

## c) Bead array technology

On bead-based chips, 100–200 ng of total RNA from each sample were polyadenylated and reverse transcribed to cDNA. A single miRNA-specific oligo (MSO) was used to assess each miRNA on the panel. All MSOs were hybridised to the sample in parallel and a solid phase primer extension step further increased sensitivity and reduces noise. Universal amplification of extended products was performed creating fluorescently labelled products identifiable by their unique MSO sequence. These labelled assay products reflected the relative abundance of the miRNAs in the sample (46). The address sequence from each MSO was used to hybridise specific miRNA products to specific locations on the Bead array substrate for readout. The Bead array reader measured the signal intensity at each address location corresponding to the quantity of the respective miRNA in the original samples. This method was fast, highly sensitive and specific with improved accuracy and flexibility as additional miRNA capture beads can be added to the mixture, allowing detection of newly discovered miRNAs (47, 48). However, it required enrichment of small RNAs by fractionation, a step that may introduce bias (49). This approach was utilised for screening differentially expressed miRNAs in the plasma samples from patients with heart failure (50). Interestingly, the authors performed extensive validation and reported several discrepancies between the levels of miRNA expression as measured by micoarrays and qPCR.

## d) Microfluidic microarrays

Microfluidic primer extension assays (MPEA) were used for routine miRNA screening. Following the hybridisation step, the isolated RNA was labelled on-chip using the "klenow" fragment of DNA polymerase I. The enzyme was added directly into the channels of a Biochip for specific elongation and labelling of hybridised miRNAs. Thus, there was no need for a labelling step before hybridisation, and no amplification was required. The volume of input RNA was very low because all reactions took place on the Biochip (51). This assay combined conventional hybridisation and an enzymatic elongation step making it extremely sensitive and allow-

 

ing miRNA profiling from as little as 20 ng of total RNA. This platform was recently applied for profiling of circulating miRNAs in patients with coronary artery disease (25).

### e) Amplification-based arrays

To increase the sensitivity and specificity of miRNA profiling, qPCR based platforms were developed. These arrays were based on a very simple principle. Total RNA was reverse transcribed using a pool of miRNA specific stem-loop primers (reverse transcription [RT] primers) in a multiplex reaction. The stem-loop structure provided specificity for only the mature miRNA target and formed a RT primer/mature miRNA chimera that extends the 3' end of the miRNA. The cDNA template was then loaded onto the 384 well fluidic card that contains a panel of qPCR primers for the same miRNA targets. A qPCR reaction was run on a thermocycler and the amplification values were calculated for the entire set of miRNAs. A preamplification step can be included to enhance the sensitivity of the assay. This approach has been widely used for profiling circulating miRNAs, and its sensitivity was further demonstrated in miRNA pools that contain only a small fraction of circulating miRNAs, i.e. HDL (8, 14, 24, 27). Moreover, it seems to have higher sensitivity than microarray-based assays, while a lower false positive rate of differential miRNA expression was observed in the qPCR arrays compared to microarrays (52). A modified version of this platform using LNA primers instead of stem-loop primers has recently become commercially available and was used to screen plasma and plasma fractions (12). The sensitivity was significantly higher than the platform using stem-loop primers. However, it also generated more false positives (almost 10%) in the no-RT control compared to no false positives in the step-loop platform. Thus, the LNA primer based qPCR profiling appears to be less specific (33).

## V. Quantification of circulating miRNAs

QPCR is the method of choice for validating the data derived from miRNA profiling. Due to the unique characteristics of miRNAs, designing a reverse transcription system to convert the miRNAs into cDNA requires (i) high specificity to ensure reverse transcription of mature miRNAs but not their precursors or genomic DNA, (ii) precision to discriminate among related miRNAs that differ by a single nucleotide and (iii) high sensitivity.

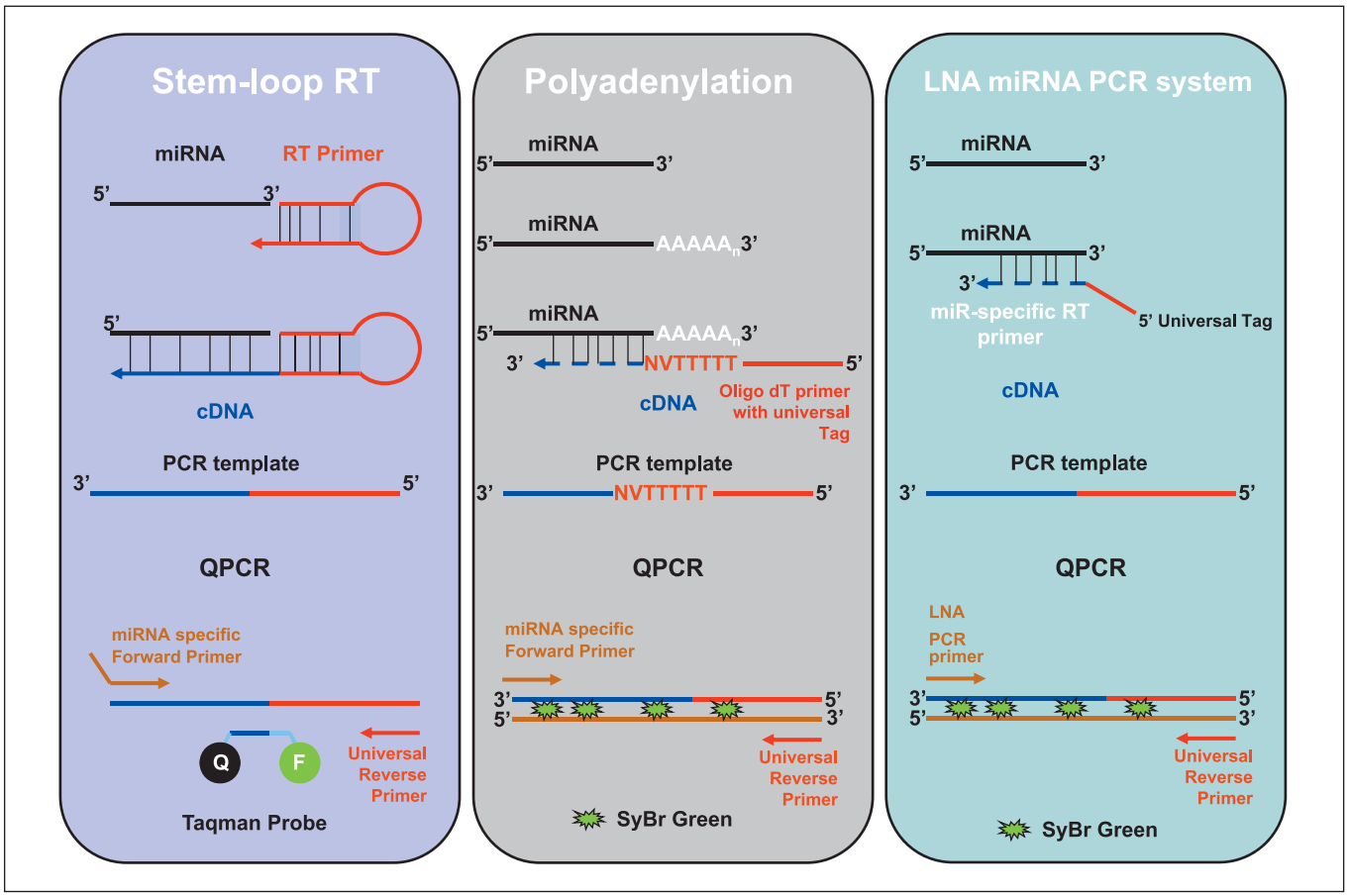


**Figure 1: The three most commonly used methods for reverse transcription of miRNAs and qPCR assessment.** left: Stem-loop reverse transcriptase primers; middle: Poly(A) tailing of mature miRNA; right: Locked nucleic acid (LNA) RT miRNA PCR.

### a) Stem-loop reverse transcription primers

This methodology involves the use of stem-loop RT primers and Taqman technology. The stem-loop structure forms a RT primer/mature miRNA chimera that extends the 3' end of the miRNA (►Fig. 1). The resulting longer RT product presents a template suitable for the standard Taqman qPCR. This includes a miRNA specific forward primer, a reverse primer and a dye-labelled Taqman probe (53, 54). Compared to conventional linear primers, stem-loop RT primers show better specificity and sensitivity probably due to the base stacking and spatial constraint of stem-loop structure. This may also prevent binding to double-strand genomic DNA molecules (53). Pools of stem-loop primers are now available as multiplex assays that facilitate the simultaneous reverse transcription of multiple mature miRNAs and enable high-throughput detection. To increase detection sensitivity, an additional step of preamplification of the RT product can be included. Typically, this consists of 10–14 cycles of PCR amplification using preamplification primers, a forward primer specifically designed for the target miRNA and a reverse universal primer. Detailed analysis comparing results generated with or without preamplification demonstrated that although there is an extremely high correlation for the most abundant miRNAs, variation is observed for low abundant miRNAs (54). Importantly, the directionality of differential gene expression is consistent, suggesting that preamplification products are suitable for the analysis of relative miRNA expression. Further studies confirmed that the systematic bias does not prohibit the comparison of relative microRNA amounts between samples (55).

### b) Poly(A) tailing of mature miRNA

In a different approach, polyadenylation of mature miRNAs by poly(A) polymerase and reverse transcription into cDNA using oligo-dT primers was performed. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNAs (►Fig. 1). This system is used in combination with SYBR Green to enable sensitive and specific quantification of mature miRNAs by qPCR. The combination of polyadenylation and the addition of a universal tag eliminate the risk of detecting genomic DNA (13, 42, 50).

### c) Locked nucleic acid RT miRNA PCR

LNA analogs can be incorporated to increase the specificity and detection efficiency for miRNAs in the RT reaction. With LNA technology, cDNA is synthesised using a miRNA specific RT primer and a sensitive stable reverse transcriptase (►Fig. 1). Subsequently, the cDNA is amplified by qPCR using SYBR Green. LNA nucleotides allow the use of miRNA-specific primers in both the RT and qPCR reaction (12, 33, 56). This can increase PCR sensitivity and amplification efficiency and is particularly useful in multiplexing to specifically target the mature miRNAs of interest (57). However, care must be taken with the design of LNA primers, as the number and position of LNA bases can substantially influence the outcome of the amplification and result in false positives (58, 59).

## VI. Data normalisation

To eliminate technical variability between different sample preparations, qPCR data have to be normalised. This is a critical issue and a major concern in profiling of circulating miRNAs. Due to the absence of generally accepted standards, synthetic non-mammalian miRNAs (mainly derived from *Caenorhabditis elegans*) were introduced to plasma or serum samples after the addition of the denaturing solution during the RNA extraction process (8, 12). These spike-in oligonucleotides can be measured by qPCR as normalisation controls. Nonetheless, these synthetic miRNAs are not incorporated in microvesicles or protein / lipid complexes. Thus, variations in extraction efficiency are not accounted for. Alternatively, various endogenous miRNAs and small non-coding RNA molecules that are detectable in all samples, show low dispersion of expression levels and no association with disease, have been used as internal controls (22, 50, 60, 61). Finally, the Ct average of a miRNA panel and the total protein content of microvesicles may serve as normalisation controls (14, 62). To ensure robustness of data, we recommend the use of different normalisation methods, preferably including the use of both endogenous and exogenous controls. For example, the Ct average of all miRNAs tested could serve as an endogenous normalisation control (provided that numerous miRNAs are assessed) (63), while the expression of a synthetic miRNA spiked into the samples during extraction could serve as the exogenous control. As circulating miRNAs display high expression correlation, computational analysis of the raw data following miRNA profiling represents an additional analytical challenge. The statistical approaches for high dimensional and highly correlated data and network inference algorithms in the evaluation of the global expression properties of miRNAs go beyond the scope of this review and have been discussed elsewhere (7).

## Conclusions

Recent advances in miRNA expression profiling and quantification technologies provide a unique opportunity to assess and evaluate the circulating miRNA patterns with remarkable sensitivity and specificity. However, several technical aspects and pre-analytical differences in sample collection / preparation may influence outcome. A consensus for isolation and handling of clinical samples, normalisation methods and analytical standards would improve inter-study comparability and facilitate the development of novel biomarkers.

 

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**Conflicts of interest**

None declared.

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