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The importance of standardization on analyzing circulating RNA

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Abstract

Circulating RNAs, especially microRNAs (miRNAs) have recently emerged as non-invasive disease biomarkers. Despite enthusiasm and numerous reports on disease associated circulating miRNAs, currently there is no circulating miRNA-based diagnostic in use. In addition, there are many contradictory reports on the concentration changes of specific miRNA in circulation. Here we review the impact of various technical and non-technical factors related to circulating miRNA measurement and elucidate the importance of having a general guideline for sample preparation and concentration measurement in studying circulating RNA.

1. Introduction - Overview of circulation RNA

The complex human blood circulation system is the major network for cells to obtain proper nutrients, dispose wastes, receive external signals, and to exchange information with other cells. Therefore, blood carries both functional and non-functional “packages” with a diverse spectrum of molecules from small metabolites to large molecules like proteins. The presence of cell-free DNA in circulation was first reported more than 60 years ago [1] and a number of applications, such as mutation screening to determine cancer treatment strategies, have been developed. It was not until 50 years later that cell-free RNA was observed in serum from patients with melanoma [2]. Even though no comprehensive investigation has been done on the origin and nature of these RNA transcripts in circulation, it is generally believed that most of the cell-free RNAs, like DNAs, are degraded products released during cell death associated with normal cell turnover or pathological conditions. Studies have shown that the concentrations of certain cell-free transcripts or their fragments correlate with different disease conditions. These findings raised the possibility of using the concentration changes of specific circulating RNA sequences as biomarkers for disease diagnosis [3, 4]. However due to the low concentration and high RNase activities in the extracellular space, the measurement of circulating RNA is difficult and unreliable. Thus the progress of developing cell-free RNA based applications is slow despite strong early interest. The finding of stable cell-free microRNA (miRNA) in various body fluids in 2008 rejuvenated the interest of cell-free RNA based biomarkers for disease diagnosis [5–8]. The initial studies showed that miRNAs are stably detectable in extracellular environment and their profiles are associated with different pathophysiological conditions.

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Conflict of Interest statement

The authors (Kai Wang, Inyoul Lee, David Baxter, Min Young Lee, and Kelsey Scherler) declare that they have no competing interests.

MiRNA is a class of evolutionarily conserved 19 to 22 nucleotide long regulatory RNA in cells [9–12]. It affects various cellular activities through modulating the transcriptome/proteome at the post transcriptional level by changing the stability of protein coding transcripts or attenuating protein translation [13–16]. The miRNA interacts with its mRNA targets through partial sequence complementarity usually at the 3' untranslated region [17–19]. This non-stringent binding condition results in a complex miRNA-mRNA interaction – a single miRNA frequently affects more than one mRNA and a single mRNA may be targeted by several different miRNAs. Based on the miRNA repository database, miRBase (www.mirbase.org) [20], there are more than two thousand miRNAs have been identified in the human genome.

To evade RNase digestion, the cell-free circulating miRNAs are either complexed with proteins or packaged in lipid vesicles [7, 21, 22]. Several different lipoproteins and RNA binding proteins such as HDL (high-density lipoprotein) [23–25], NPM1 (nucleophosmin 1) [26], and Ago2 (argonaute 2) [27, 28] have been shown to bind and protect miRNA from RNase activities. Extracellular lipid vesicles are membrane vesicles released by cells [29–31]. Based on their biogenesis, the extracellular vesicles (EVs) can be grouped into three major classes: 1) microvesicles, 2) exosomes, and 3) apoptotic bodies. Microvesicles are 100–1000 nm in diameter and produced by outward budding of plasma membrane [32, 33]. Exosomes are smaller vesicles about 30 to 150 nm in size and produced by inward budding of endosomal membrane. Exosomes accumulate in the multivesicular bodies (MVB) and are released to the extracellular environment once the MVB is fused with cell membrane [34, 35]. Apoptotic bodies are 50 to 5000 nm in diameter and released from apoptotic cells [36, 37]. Wide ranges of proteins, lipids, nucleic acids and metabolites have been seen in all EVs, and the content of these biomolecules often reflect the cellular origin [38, 39]. A number of studies have shown that upon take-up by cells, the molecular contents in the exosome can affect the activities of recipient cells [31, 35, 40, 41]. These findings suggest exosomes may play a role in intercellular communication.

2. Circulating miRNA as biomarker

Most of the tests for disease diagnosis and monitoring used in clinics are based on specific protein concentration changes in body fluids, for example amino transferases for liver function [42–45], prostate specific antigen (PSA) for prostate cancer [46–48], and troponin for myocardial infarction [49, 50]. Despite devoting significant amounts of resources for new protein biomarker discovery, very few successful examples have been reported. This is due to factors such as the complex post-translational modifications associated with proteins, difficulties to develop high affinity capture agents with sufficient specificity, and the low concentration of protein of interest in body fluids.

The discovery of disease-associated concentration changes of specific extracellular miRNA such as miR-141 for prostate cancer [5, 51, 52], miR-122 for liver related diseases [6, 53–55], miR-208 and miR-499 for cardiovascular diseases [56–59] in body fluids raised the interest of developing a circulating miRNA-based biomarker for disease diagnosis. In comparison to protein-based biomarkers, miRNA has several advantages including a much smaller repertoire, easier to develop assays with sufficient specificity, and an amplifiable

detection signal. However, circulating miRNA measurement suffers from consistency and reproducibility issues when comparing the results from different studies. This is in part due to low RNA concentration, short length of the miRNA sequences, high sequence similarity among miRNA family members, and sequence length variations. Furthermore, a number of studies indicate other issues that may affect the miRNA measurement results. These include technical and non-technical factors such as sample type, storage condition, RNA isolation method, measurement platform and the donor's physical state. This review will address the impact of some of these factors on miRNA measurement and illustrate the importance of having a general guideline on how samples are prepared for miRNA concentration measurement.

3. Tools to profile and measure the RNA in circulation

Even though there are several platforms for miRNA measurement such as quantitative polymerase chain reaction (qPCR), microarray, and next generation sequencing (NGS), it is still quite challenging to accurately measure miRNAs, especially circulating miRNAs. Each method has its strengths and weaknesses that need to be considered depending on the nature of the research. The qPCR platform relies on reverse-transcription (RT) of miRNAs to complementary DNAs, followed by polymerase chain reaction (PCR). The RT of mature miRNA can be primed by addition of a poly (A) tail [60] or by generation of a RT primer binding site using a stem-loop primer that binds to specific miRNA [61, 62]. The qPCR platform has been the method of choice to measure specific miRNAs due to its sensitivity and the ease of use, but it has disadvantages as well, such as possible cross amplification and low throughput. Quantitative PCR has routinely been used to validate miRNA profiling results obtained from platforms such as microarrays and NGS. Microarray platforms are a well-established hybridization-based method commonly used for RNA profiling, and are provided by several manufacturers. Microarrays are an economic, high-throughput method that have been successfully used to profile miRNAs from cells and tissues, but may not be suitable for circulating miRNA analysis due to the low RNA concentration in body fluids especially when the sample volume is a limiting factor. The cross-hybridization due to sequence homology within miRNA families, short probe length, and inability to distinguish between unprocessed and mature miRNA can cause some issues with microarray data analysis. Also, normalization of microarray data relies on the assumption that total miRNA levels are consistent between samples. Due to RNA extraction variations and unreliable of RNA concentration measurement methods, this is rarely true [63, 64]. The NGS method is an emerging technology that has recently become a preferred method for circulating RNA analysis due to its lower cost, higher throughputs, and lower RNA input compared to other methods. In addition, measuring miRNAs with NGS can avoid some of the challenges faced by microarray and qPCR. For example, the short length of the miRNA itself and sequence similarities become less of an issue in NGS-based profiling. The NGS results provide an opportunity to identify novel miRNAs and discriminate isomiRs in addition to quantifying known miRNAs. However, NGS-based methods suffer from drawbacks including requiring bioinformatician support for data analysis, tedious sequencing library construction processes, and sequence bias resulting from the sequencing library construction process [65, 66].

4. Factors affect the spectrum of extracellular RNA

There are a number of factors that can have a substantial impact on the results of circulating miRNA profiling. Some of these are sample related such as sex and state of the subject, sample collection time, and type of samples. Others are experiment related including sample processing, sample storage condition, RNA extraction method, as well as choice of measurement platform. We will briefly review the effects of some of these factors (summarized in Table 1).

4.1. Sample related factors

4.1.1 Sample type—It is expected that different types of biofluids have different spectra of extracellular RNA. However, there is some confusion about the difference between serum and plasma – both are derived from blood. The difference is whether anti-coagulant was used while drawing blood anti-coagulant was not used in serum preparation. The miRNA identified from either serum or plasma are often compared and grouped together in literature. We and others have previously shown that the choice of sample types can have substantial impacts on the spectrum of extracellular miRNA in blood [67, 68]. Using plasma and serum samples from the same individuals, we observed higher total RNA concentrations in serum samples compared to the plasma samples. The increase of RNA concentration in serum may be due to RNA released by blood cells and platelets during the blood coagulation process. No correlation between the concentration of RNAs and the number of detected miRNAs was observed between serum and plasma in our study with a limited number of samples. In addition, the list of the most abundant set of miRNAs detected in serum and plasma is similar. McDonald et al. [68] showed that the concentrations for miR-15b, miR-16, and miR-24 were higher in plasma compared to serum. Therefore, it is critical to compare circulating miRNA changes within the same sample type since it is possible that the disease-associated cell-free miRNAs identified are different between serum and plasma.

4.1.2. Contamination – platelet and blood cells—Researchers also assess factors such as cell lysis on the spectrum of circulating RNA. Kirchner et al. [69, 70] studied the impact of hemolysis and showed that the concentration of endogenous miR-16, a miRNA often used for normalization, is one of the most abundant miRNAs present in RBCs. Concentrations of miR-16 were higher and more variable in hemolyzed plasma or serum samples. Therefore, miR-16 cannot be used as a normalizer with hemolyzed samples. Platelets also contain large numbers of miRNA, which causes a difference between the profile of circulating RNA between plasma and platelet poor plasma. Prichard et al. [71] demonstrated that the platelet-derived miRNAs have a significant influence in the miRNA spectrum in plasma and serum. Cheng et al. [72] showed that centrifugation force used in plasma processing affected levels of circulating miRNAs due to the amount of platelets remaining in the sample. Therefore, caution must be taken when comparing plasma circulating miRNA measurements from different studies.

4.1.3. Gender and sample collection time—The physiological and genetic differences between genders can have a significant effect on the biological composition of body fluids. Circulating miRNA has been implicated as a biomarker for various disease conditions;

however, disease prevalence and outcome can be affected by gender. We and others showed that gender can affect the spectrum of circulating RNA [73, 74]. Recent reports indicate that there are some circulating miRNAs showing gender specific disease association. For example, let-7g and miR-221 levels showed a positive correlation with the progression of metabolic diseases in female patients [74]. Additionally, events like menstrual cycles [75–78] and pregnancy [79, 80] also altered the overall circulating miRNA profile. For example, the levels of miRNAs from a chromosome 14 and 19 miRNA clusters significantly increased during pregnancy [81]. Therefore, sex difference may affect the discovery of disease-associated circulating miRNA and needs to be considered in study design.

Even though there has been no large-scale comprehensive investigation, there are indications that the spectrum of circulating RNA, like body temperature, showed a diurnal effect [82]. A number of biological systems are affected by the circadian cycle; therefore it is not surprising that these activities affect the “release” of RNA into the extracellular environment and alter the profile of circulating RNA. For example, Shende and coworkers [83] showed that the circulating miR-494 and miR-152 levels displayed diurnal oscillations in mice. Heegaard et al. [84] also demonstrated that 26 out of 79 detectable miRNAs in plasma exhibit a rhythmic behavior and are distributed in two main phase patterns in young and healthy male subjects. These findings illustrate the importance of considering bio-oscillations in miRNA biomarker studies and that the sample collection time should be recorded and included in scientific reports.

4.1.4. Exercise and diet—We and others have shown that RNA molecules from exogenous species contribute to the global RNA profile in circulation [85] although some researchers reported that exogenous RNA including plant miRNAs observed in humans could be environmental contaminants or artifacts [86, 87]. These exogenous RNAs may have no other biological activities besides nutritional value [88]. MacLellan et al. [89] showed that the number of detectable miRNA is higher in samples from non-fasting subjects. Therefore, food intake may affect the circulating RNA profile similar to its effect on the level of glucose and lipoproteins in circulation. The impact of food intake on circulating RNA may be due to higher levels of miRNA carriers like HDL, and elevated metabolic activities which may induce changes of circulating miRNA profile and RNA molecules from food consumed. Due to the high sequence conservation of some miRNAs across different species, there is a possibility that some of the miRNA in circulation may not be derived from cells in the body but from exogenous sources. For example miR-21-5p, a miRNA associated with cell proliferation and cancer progression, is conserved in vertebrates; its sequence is identical between turtles (aca-miR-21-5p), snakes (oha-miR-21-5p), bovines (bta-miR-21-5p) and humans (hsa-miR-21-5p).

We and others also demonstrated the impact of exercise on miRNA profile in circulation. In an animal model, we showed concentration changes of muscle-enriched miRNAs like miR-133 family members in circulation after exercise. Similar findings have also been observed in athletes. For example, the levels of miR-1 and miR-133a are significantly increased in circulation for people who completed a marathon race [90]. Type of exercise also affects the change of circulating miRNA [91]. Uhlemann et al. [92] showed that the changes of miR-126 and miR-133 levels in circulation depend on the type of exercise. In

healthy individuals who finished a marathon, both circulating miR-126 and miR-133 levels increased, but only miR-133 levels increased in individuals with resistance exercise and only miR-126 in either maximal spiroergometry exercise or 4 hours of bicycling. This difference could be caused by different tissue damage associated with different types of exercise, such as endurance exercises causing endothelial damage (miR-126 is enriched), and resistance exercise that has an effect only on the muscles (miR-1 and miR-133 are enriched) [93].

4.2. Experimental procedure related factors

4.2.1. Sample processing—The processing of biofluid samples also impacts the profile of RNA. One example is the types of anticoagulant including K₂ ethylenediaminetetraacetic acid (EDTA), Na₃ citrate, and heparin used in plasma preparation. It is well-known that heparin inhibits the activities of reverse transcriptase and DNA/RNA polymerases used in various RNA measurement methodologies including RT-PCR, NGS, and microarray [94, 95]. Therefore heparin-treated plasma will affect the measurement results and is not suitable for circulating RNA analysis. Heparinases can be used to remove heparin in the sample, but the use of heparinase can introduce additional experimental variability. In addition, the use of heparinase significantly increases the cost of RNA measurement. Recent studies also suggest that citrate treated plasma shows lower miRNA concentrations based on qPCR measurement compared to EDTA treated plasma from the same individual [56, 96].

4.2.2. Sample storage time and condition—Once the samples have been prepared, the storage condition can also affect the circulating RNA profile. For example, McDonald et al. [68] showed that the levels of several miRNAs including miR-16 increased from 24 to 72 hours in either ambient, 4°C or –20°C. The increase of miRNA concentration over time is unexpected. There are two possible explanations: one is the release of miRNAs from platelets or lipid vesicles during storage. The other is a conversion between different forms of miRNA packages such as between an RNA binding protein-RNA complex and a lipoprotein-RNA complex, and different types of RNA packages may have different RNA extraction efficiency.

4.2.3. RNA extraction method—A number of reports have investigated the effect of RNA isolation methods on miRNA measurement. Although there is no conclusion on which method is the best, there is a consensus that different RNA isolation methods affect the results of circulating RNA measurement. For example, Li et al. [97] compared miRNA measurement results including RNA yield and amplification efficiency of plasma RNA using seven different commercial kits: RNAdvance (Agencourt Bioscience, Beckman Coulter, Beverly, MA), MAGMAX (Life Technologies, Thermo Fisher Scientific), miRCURY-Biofluids (Exiqon, Vedbaek, Denmark), Quick-RNA (Zymo Research, Irvine, CA), DirectZol (Zymo Research, Irvine, CA), miRNeasy (Qiagen, Hilden, Germany), and mirVana (Thermo Fisher Scientific). As observed, some kits yield very little amounts of plasma miRNAs. The miRNeasy kit seems to show a better overall performance; however, this finding needs to be verified in different laboratories with a larger number of diverse samples.

Tanriverdi and coworkers [98] also demonstrated different performances of four RNA isolation methods including an in-house protocol for qPCR based extracellular RNA measurement. They reported that there are significant differences in RNA yields and amplification efficiencies among different RNA isolation and measurement methods for low-abundant miRNA detection from plasma. Therefore, it is critical to report exactly how RNA was isolated from biofluid samples in studies.

4.2.4. Measurement platform—It is often observed that there is poor agreement among results obtained from different platforms [64, 99–102] or even from the same technology platform but using products from different vendors [64, 103–106]. Studies have reported that some miRNAs showed platform dependent variations in measurements. For example, Tan et al. [104] evaluated three qPCR systems (TaqMan, miScript, mirCURY) and found different accuracy, sensitivity and reproducibility among the three methods. Farr and colleagues [99] also systematically compared four different qPCR based miRNA profiling platforms (standard 96-well qPCR panel, 384 microfluidics PCR panel and two ultra-high content microfluidic platforms – OpenArray and Fluidigm) for circulating miRNA detection, and evaluated platform variability and reproducibility. The authors showed that technical factors can influence the results from miRNA qPCR data, such as the sensitivity to measure low abundant miRNAs that are inversely related to qPCR reaction volume. NGS based miRNA profiling has gained significant interest in recent years. Nearly all common commercial kits rely on T4 RNA ligases to ligate single-stranded adapter sequences to the 3′ and 5′ ends of miRNAs. However the most commonly used kits from Illumina and New England BioLabs are affected with significant bias and some miRNAs can only be efficiently observed in sequencing results from one of the library construction kits [107, 108]. It is believed the majority of bias is introduced during the ligation steps. Choice of ligase can have a large effect on the yield and makeup of miRNA in the library, as well as amount of undesirable side-products such as adapter dimer. Newly developed modified ligases can reduce the formation of side products and increase library yield [66]. However the sequence of the adapters themselves can also affect the resulting miRNA profile, which is likely due to preferential ligation based on both the miRNA and adapter sequences. It has been shown that adding degenerate bases at the appropriate ends of the 3′ and 5′ adapters can increase ligation efficiency and reduce sequence-specific ligation bias [109]. This allows individual miRNA to form more stable secondary structures with the adapters and to ligate more efficiently. These changes, in addition to optimizing the concentration of polyethylene-glycol, can greatly reduce the ligation bias and increase the number of miRNA detected. However a more comprehensive comparison of different NGS library construction methods is needed in order to document the differences of miRNA profiling results associated with different library construction methods.

Mestdagh et al. [103] compared 12 different commercially available platforms including qPCR, microarray, and NGS, and found substantial inter-platform differences when evaluating differential miRNA expression. This creates some potential issues with miRNA analysis since different technologies are often used for validation purposes. In addition for samples having lower total miRNA levels, such as biofluid samples, intra-platform reproducibility suffers and inter-platform variation tends to increase [63, 101].

4.2.5. Data processing and normalization—Analysis of miRNA measurement results can be summarized into three major steps: preprocessing – to remove low quality data points, normalization – to remove sample-to-sample variations, and analysis - to identify affected miRNAs. For NGS data, the adapter sequences and low-quality reads need to be removed before the reads are aligned to reference sequence databases. The normalization step is important, since subsequent analyses, such as the detection of differential expression, are highly dependent on the normalization method. Unlike gene expression analysis, there is no housekeeping or invariant miRNA that can be used as an internal control. A set of nonhuman spike-in RNAs is commonly used for normalization in miRNA measurement, especially for qPCR based profiling. For miRNA microarray, some methods such as global mean or quantile normalization that are commonly used for gene expression microarray, are adapted for miRNA analysis. Most of these normalization methods rely on the assumption that total miRNA levels are consistent between samples, but due to RNA extraction variations and unreliable measurement methods, this is rarely true [63, 64].

For sequencing based profiling, among the several normalization methods that have been proposed, counts-per-million (cpm) is widely used. However, studies have shown that this is insufficient to remove technical variations across samples. This is due to the random sampling nature of the NGS technology and therefore normalizing based on cpm (or library size) will not correct the variation. Recently, Tam et al. compared several normalization methods using a spike-in dilution and publicly available miRNA sequencing data with matched qPCR data [110]. The authors compared eight different normalization methods (counts-per-million, total count scaling, upper quartile scaling [111], trimmed mean of M [112], DESeq [113], linear regression [114], cyclic loess and quantile [114] with regard to the normalized miRNA count distribution, variance, bias, and accuracy of differential expression analysis. From the spike-in dilution data of 12 *Arabidopsis thaliana* miRNAs and human reference miRNAs, they concluded that cpm and total count scaling are inadequate in all aspects. Data normalized by upper quartile scaling (UQ) or trimmed mean of M (TMM) showed high sensitivity and specificity. In addition, data normalized by UQ and TMM have smaller variances, especially for low abundant species while cpm and total count scaling increased variance across all abundance levels. Reduction in fold-changes is also observed in cpm, total count scaling and quantile normalization. Using publicly available sequencing data with matched qPCR data, the authors concluded that using one mismatch with UQ or TMM normalization leads to more accurate results in downstream analyses.

5. Summary

The search for informative diagnostic and prognostic markers based on non-invasive or minimally invasive techniques is at the forefront of many disease-oriented studies. Extracellular miRNAs from body fluids such as serum, plasma and urine are emerging as biomarker candidates even though the development of extracellular miRNAs as biomarkers is still in its infancy. While an abundance of studies report differential concentrations of specific circulating miRNAs associated with different disease conditions, important procedural information such as sample collection time and donor status most of the time is lacking and needs to be recorded and disseminated to the research community. Importantly, attention to the fasting status of the patient and blood collection procedures, such as

anticoagulants and needle size, should be considered when profiling extracellular miRNA from blood. Extraction methods vary among reports; however, information on the starting fluid volume and especially the efficiency of extraction is often missing. The availability of this information will enable investigators to standardize procedures and perhaps compare profiles across various patient cohorts.

6. Conclusion and suggestions

The field of circulating miRNA is less than 10 years old; however, significant technical improvements have been made during this short period. These include the development and adaptation of the NGS platform in circulating RNA research, the development of informatics tools to map and analyze small RNA sequence reads, recognition of the compartmentation of RNA in circulation, and development of commercial kits to assist various experimental steps. This short review did not cover the growing field of RNA in EVs (extracellular vesicles) and issues associated with EV purification. The isolation, profiling, and functional exploration of EVs will be an exciting field in the next few years. However, factors affecting circulating RNA analysis may also impact the characterization of EVs in circulation.

Despite the fact that we do not fully understand all the factors that may affect the spectrum of circulating RNA, there is ample evidence to suggest that circulating RNA, especially miRNA, may play an important role in disease diagnosis. Even though significant improvements have been made in the past few years on studying circulating RNA, the field like other newly developed discipline, lacks consistency with numerous contradicting reports. The low concentration of RNA in nuclease-rich extracellular environment also contributes to the variations of circulating RNA measurement. It is clear that comprehensive studies are needed to document and understand factors – both technical and non-technical – that affect the profile of circulating RNA, which may help to reduce some of the inconsistent findings. We strongly encourage to fully document and report all the sample collection and experimental details for current studies before a standard or general guideline can be set. The information to be collected and reported should at least include the condition of the subjects, sample collection time, sample processing procedure, RNA extraction method, RNA profiling details, and the data analysis procedure (Table 2). This would allow a more meaningful comparison and integration of data from different studies, which may facilitate the development of circulating RNA based clinical applications.

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Key points

- Circulating microRNAs have been emerged as non-invasive disease biomarkers; however, there are many contradictory reports.
- Studies have shown that both technical and non-technical factors may affect the spectrum of circulating RNA. Comprehensive studies to identify and document these factors are urgently needed.

Summary of factors that affect measuring levels of circulating microRNAs described in this article.

Table 1

Factors	Source	Note	Reference
Sample type	Serum vs plasma	Higher miRNA concentrations in serum samples compared to the plasma	[67]
	Serum vs plasma	Levels of three miRNAs (miR-15b, -16, -24) were increased in plasma by qRT-PCR in healthy individuals	[68]
Contamination	Platelets and cells	Concentrations of miR-16 were higher and more variable in hemolyzed samples	[69, 70]
	Platelets	Platelet-derived miRNAs have significant influence on circulating miRNA profile	[71]
	Platelets	Centrifugation force used in plasma processing affected levels of circulating miRNAs due to the amount of platelets remaining in the sample	[72]
Sample related factors	sex	Gender affects the concentration of some circulating RNAs (the levels of miR-548-3p, -1323, -940, -1292 are increased in females)	[73]
	sex	let-7g and miR-221 levels showed a positive correlation with the metabolic disease progression in female patients	[74]
	pregnancy	Levels of primate specific and placenta enriched miRNAs significantly increased during pregnancy	[81]
	circadian cycle	Circulating miR-494 and miR-152 levels displayed diurnal oscillations in mouse	[83]
	circadian cycle	26 out of 79 detectable miRNAs in plasma exhibit a rhythmic behavior and are distributed in two main phase patterns in young healthy male subjects	[84]
Diet and Exercise	fasting, smoking	The number of detectable miRNA is higher in samples from non-fasting subjects	[89]
	Exercise	The changes of miR-126 and miR-133 levels in circulation depend on the type exercise	[92]
Sample processing	Anticoagulant	Heparin inhibits the activities of reverse transcriptase and DNA/RNA polymerases used in various RNA measurement methods	[94, 95]
	Anticoagulant	Citrate treated plasma showed lower miRNA concentration compared to EDTA treated plasma	[56]
Sample storage time and condition	Storage time and temperature	The levels of several miRNAs increased over time (from 24 to 72 hours) at room temperature, 4 and -20 degree.	[68]
RNA extraction method	Commercial kits	Different RNA isolation method affects the miRNA measurement result	[97]
	In house kit vs commercial kits	Compared different RNA isolation methods including an an-house kit that performed better than three commercial kits tested	[98]
Measurement platform	qPCR vs microarray, NGS	Interplatform inconsistency especially results from differential analysis	[103]
	qPCR systems	Different qPCR kits (TaqMan, miScript, mirCURY) were tested using BioMark platform	[104]
	qPCR vs RNA-seq	Compared four different qPCR based miRNA profiling platforms and RNA-seq	[99]

Factors	Source	Note	Reference
	Microarrays	Intra-platform irrepeatability and inter-platform variation increased with lower total miRNA levels	[63]
	Sequencing library preparation	Modified ligases can reduce the formation of side products and increase library yield	[66]
	Sequencing library preparation	Adding degenerate bases at the appropriate ends of the adapters can increase ligation efficiency and reduce sequence specific ligation bias	[109]
Data analysis			
	Data normalization method	Small RNA mapping and normalization method affect the results of differential analysis	[110]

Table 2
List of information that should be included in circulating RNA measurement related scientific reports.

Category	Example of information to be included
Type	Serum or plasma
Collection time	Sample collection time
Processing detail	Centrifugation speed for serum or plasma preparation.
Storage condition	−80, −20, 4 °C
Gender and age	Male or female
Donor condition	For example, fasting or not
RNA isolation	How the RNA was isolated
Measurement platform	Should include reagent vender and detailed protocol such as qPCR condition and sequencing library construction method.
Data processing	How the data were processed and normalized.