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TechNote - Minimum Preanalytical Information for the Publication of Studies on Circulating Cell-Free microRNA-based Biomarkers

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ABSTRACT

Circulating cell-free microRNAs (miRNAs) are emerging as promising biomarkers with broad potential for clinical applications. However, pre-analytical variability significantly affects miRNA quantification and hampers reproducibility across studies. This work aims to define the Minimum Preanalytical Information required for the publication of studies on circulating cell-free miRNA-based biomarkers. We review key pre-analytical factors that influence circulating miRNA levels quantified using RT-qPCR. Critical variables include blood collection timing, sample type, centrifugation protocols, transport and storage conditions, hemolysis, lipemia, medication, physical activity and pathogen inactivation methods. We introduce a standardized checklist to promote methodological transparency and inter-study comparability. The final aim is to enhance the reliability of miRNA-based biomarker research and support its successful translation into clinical practice.

Keywords
Biomarker; microRNA; pre-analytical variables; RT-qPCR

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Background

Circulating cell-free microRNAs (miRNAs) have emerged as a promising tool with potential applications in clinical decision-making [1]. Their accessibility through non-invasive or minimally invasive methods, combined with the availability of biobanked samples and measurement techniques, has facilitated the extensive exploration of these short transcripts for biomarker development.

Despite extensive research efforts, integrating miRNA-based biomarkers into routine clinical practice remains challenging. A major barrier to the successful translation of circulating miRNAs into reliable biomarkers is the lack of reproducibility across studies from different laboratories. Variability can be introduced at several stages of the process from sample collection, storage, sample processing, RNA isolation, miRNA quantification and data analysis [2].

To improve reproducibility in RNA-based biomarker research, the scientific community is actively working toward methodological standardization and the implementation of quality control measures

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[3,4]. The establishment of guidelines for best practices and standard operating procedures (SOPs) ensures consistency across studies. Additionally, transparent and detailed reporting of methods and results is critical to facilitate replication and identify potential sources of variability. The methods section of a scientific paper is pivotal in this respect, providing information about the experimental protocols. This section must include enough detail to allow readers to assess the reproducibility and robustness of the study, such as descriptions of the study population, sample types, experimental design, data acquisition and analysis protocols.

In this TechNote, we examine and discuss the pre-analytical parameters that should be detailed in the methods section of studies on circulating cell-free miRNA-based biomarkers, with a particular focus on plasma and serum samples and the gold-standard RT-qPCR methodology. This TechNote also proposes a comprehensive checklist covering the minimum pre-analytical information that should be reported in publications focused on circulating cell-free miRNA-based biomarkers (**Table 1**) [based on the MIQE guidelines [5]].

The TechNote has been developed under the auspices of the AtheroNET COST Action (CA21153, https://atheronet.eu/), which represents an international and interdisciplinary network dedicated to accelerating the utilization of multiple omic technologies to bring novel paradigms in prevention, diagnosis and treatment of atherosclerotic cardiovascular disease [6].

Minimal Preanalytical Information

Timing for Blood Sampling and Processing

The timing of blood sample collection is a key pre-analytical variable, typically governed by standardized protocols recommending an 8- to 12-hour fasting period in the early morning. This helps minimize the impact of postprandial states (e.g., lipemia) and circadian fluctuations. However, timing presents a particular challenge in studies involving acute cardiovascular events, as variability in patient admissions, treatment initiation and diagnostic procedures significantly impact the timing of sample collection (see Section 2.7 on Medication).

The interval between blood collection and subsequent processing (e.g., plasma/serum preparation, aliquoting and storage) should also be considered. Processing delays alter miRNA levels, although the degree of impact varies between individual miRNAs. A study analyzing 179 miRNAs in plasma samples processed at 30 minutes, 2, 6 and 24 hours post-collection demonstrated that processing time was a major source of variability [7]. Out of the total panel of miRNAs analyzed, 53 showed significant changes, including commonly used endogenous controls such as miR-16-5p, miR-25-3p and miR-223-3p.

To reduce variability and improve data reliability, samples should be processed within consistent timeframes using standardized protocols. This approach minimizes potential changes in circulating miRNA levels due to differences in storage time or conditions.

Sample Type

Clearly identifying the sample type is essential to account for the effects of different anticoagulants and stabilizers on miRNA quantification. Various blood collection tubes are available, including those designed for serum (with clot activator and separation gel) and plasma (e.g., EDTA, sodium citrate, heparin, oxalate). Additionally, specialized systems developed for molecular diagnostics are available. BD Vacutainer® PPT™ is a closed system allowing separation and storage of undiluted EDTA plasma in the primary blood collection tube. Other examples include the PAXgene® Blood RNA Tube, Streck RNA Complete BCT®, and cf-DNA/cf-RNA Preservative Tubes (Norgen Biotek Corp), which aim to preserve RNA integrity. Consistency on the type of collection tubes is important to avoid introducing pre-analytical variability, since tubes containing RNA preservatives will result in different stability of the RNA, even under short processing times, from those that do not.

While tubes coated with RNA preservatives may appear ideal, recent findings from the exRNAQC consortium reveal a nuanced reality. In a comprehensive study on biofluids (serum and plasma) across three different time intervals post-blood collection, the impact of blood collection tubes on downstream extracellular RNA (exRNA) sequencing was evaluated [8]. Intriguingly, preservation tubes marketed for stabilizing extracellular nucleic acids for 7 to 14 days exhibited compromised stability over time compared to non-preservation tubes. The study concluded that the tested preservation tubes are unsuitable for exRNA analysis within the examined time intervals. Citrate tubes were recommended for extracellular analysis and pro-

Table 1 | A comprehensive checklist for the publication of circulating cell-free miRNA-based biomarker studies.

Item	Description	Level of Importance
1. Timing for Blood Sampling and Processing	Specify the time of day when samples were collected and the time span for blood sample processing	1
2. Sample Type	Clearly describe the type of sample and blood collection tubes	1
3. Centrifugation Protocol	Provide information on the centrifugation protocol	1
4. Sample Transport and Storage	Detail the conditions and procedures for sample transportation Specify the conditions and duration of storage Detail the number of freeze-thaw cycles	2
5. Hemolysis	Report on the assessment and presence of hemolysis	1
6. Lipemia	Report on the assessment and presence of lipemia	1
7. Medication	Document the use of medications taken by sample donors	2
8. Exercise	Record physical activity prior to blood sampling	3
9. Sample Pretreatment	Detail the specific procedures applied to biological samples	1

This checklist is designed to delineate the minimum pre-analytical information that should be addressed and reported in the Methods section of publications reporting results from circulating cell-free miRNA-based biomarker studies. This checklist was developed based on the MIQE guidelines [5].

cessing tubes within four hours of blood draw were advised for exRNA analysis.

The choice of blood derivatives for miRNA investigation significantly influences study outcomes, as evidenced by the fact that different blood derivatives yield distinct profiles. Serum and plasma miRNA content differs due to the release of miRNAs during the coagulation processes in serum, limiting comparisons between these sample types [9]. Regarding blood processing timing (see Section 2.1), evidence indicates that, for certain miRNAs, variance due to delayed sample handling may be partially attributed to *in vitro* platelet activation [10]. Furthermore, differences in hemolysis levels have been observed based on the type of anticoagulant used, with notable variation reported among EDTA, citrate and CTAD [10].

It is important to note that heparin interferes with PCR reactions [11], as it binds calcium and magnesium ions, essential components of the PCR master mix. Consequently, when isolating RNA for RT-qPCR, heparin-containing tubes should be avoided [12]. Citrate, on the other hand, may dilute plasma and potentially increase hemolysis.

Centrifugation Protocol

Providing a detailed description of the centrifugation conditions used to obtain plasma or serum is critical to ensure reproducibility and precision in miRNA research. Protocols for obtaining serum, plasma or platelet-poor plasma (PPP) vary widely among laboratories, especially in clinical settings. The process of obtaining plasma (platelet-rich plasma; PRP) typically involves centrifugation of whole blood at 1,200 to 2,000 g for 10 to 15 minutes. For PPP, an additional centrifugation step is required, with protocols varying in duration (10 to 20 minutes) and speed (2,000 to 5,000 g). Establishing the optimal protocol for obtaining plasma prevents contamination by platelet-enriched RNAs. Platelets, known to harbor a significant amount of RNAs, release these transcripts during activation and destruction, causing notable changes in the transcriptomic landscape. Indeed, Krammer et al. [13] demonstrated that miRNA profiles in PRP cluster closely with those in platelets. Additionally, the study of Mitchell et al. [14] showed that freeze–thaw cycles influence plasma miRNA profile, if not prepared as PPP. The reason for this is that miRNAs are released from the residual blood cells and contaminate the sample [15].

Efforts have been made to standardize centrifugation procedures. The International Society on Thrombosis and Haemostasis [16] defined the protocol for obtaining PPP. Whole blood samples should be double-centrifuged: firstly, at 2,500 g for 15 min at room temperature. Afterwards, plasma should be collected in a plastic tube, leaving 1 cm of plasma above the buffy layer and cautiously handled to avoid disturbance and centrifuged for a second time at 2,500 g for 15 min at room temperature and collected afterwards into a new plastic tube, leaving approximately 100 µL at the bottom of the plastic tube. For miRNA analysis, Chan et al. [17] propose a dual-spin protocol for processing PPP: an initial spin at 1,500 g for 15 minutes followed by a second spin at 2,500 g for 15 minutes, both conducted at room temperature. While some protocols suggest a second centrifugation at 10,000 (or even 16,000) g, the feasibility of employing such high-speed centrifuges in clinical settings may be limited. In summary, careful consideration of centrifugation parameters ensures rigorous blood processing protocols.

It is also important to consider the impact of centrifugation parameters when specific miRNA carriers, such as lipoproteins, are being analyzed. Lipoproteins share biophysical properties with exosomes, which complicates the interpretation of miRNA profiles [18].

Sample Transport and Storage

Transporting samples is a significant aspect, particularly when blood collection is conducted at clinics distant from the research center or in multicenter studies with samples sent from several centers to a central facility. Transport extends the time before blood processing, increasing the risk of introducing variability if not managed under proper conditions [19]. Glinge et al. [20] examined the effects of physical disturbance during transport on miRNA stability. Their study found that one hour of disturbance did not affect miRNA levels, but eight hours of disturbance led to a decrease in miR-1-3p and miR-21-5p levels in separated plasma and in miR-21-5p levels in serum whole blood. This highlights the importance of minimizing prolonged physical disturbance during transport to maintain the integrity of samples.

RNA integrity relies on careful management of storage conditions. RNA is highly vulnerable to degradation from RNase activity, making immediate processing or appropriate storage essential. While cooling samples to 4°C temporarily slows enzyme activity, it is important to note that this temperature could activate platelets. For long-term preservation of RNA, storage at -80°C is considered the gold standard, particularly for extended periods. However, this presents challenges in clinical settings where access to this type of freezers may be limited.

In a study conducted by Chan et al. [17], the stability of miRNAs in serum samples was rigorously assessed under various storage conditions. Samples were stored at 25°C for 3 days, 4°C for 3, 7 and 30 days, -20°C for 3, 7, 30, 90, 180, 270 and 360 days and -80°C for 3, 7, 30, 90, 180, and 360 days. The findings indicated long-term storage at -80°C significantly extended miRNA stability, though an exception was observed in whole blood samples stored for nine months. In contrast, plasma samples demonstrated stable miRNA levels, even with extended storage. Freeze-thawing up to four cycles affected only one out of eight miRNAs [21], suggesting that freeze thawing should be avoided since the effect will not uniformly affect all miRNAs.

To mitigate these stability issues, it is recommended that plasma or serum samples be aliquoted before being stored at -80°C. This prevents repeated freeze-thaw cycles, which degrades miRNA. Proper aliquoting and storage practices ensure that samples remain intact and suitable for reliable miRNA analysis even after extended periods.

Hemolysis

Hemolysis, resulting from the rupture of red blood cells (during blood collection and processing), is a confounding factor that alters the circulating miRNA pool. This disruption releases a large quantity of intracellular RNA into the plasma or serum, leading to potentially misleading results in miRNA analysis. miRNAs that are highly concentrated in red blood cells, including miR-20b-5p, miR-363-3p and miR-451, serve as effective biomarkers for detecting hemolysis and assessing sample contamination [17]. Their presence in plasma or serum may not reflect true extracellular levels but rather contamination due to hemolysis.

In light of this, it is important to include routine checks and, whenever possible, quantification of hemolysis levels [22]. The method employed for hemolysis monitoring and whether hemolysis affects the miRNA candidate should be disclosed in the publication. As the field advances, continuous attention to hemolysis assessment will undoubtedly enhance the robustness of pre-analytical considerations in miRNA studies.

Lipemia

There is a notable lack of studies that comprehensively evaluate the impact of lipemia on circulating miRNAs. Lipoproteins, which

act as important extracellular carriers of miRNAs [23], could influence the circulating miRNA profile, particularly under conditions of elevated lipoprotein levels, such as the postprandial state. Among them, high-density lipoprotein (HDL) particles play a pivotal role contributing significantly to the regulation of the circulating miRNA landscape [24]. Recent evidence further indicates that HDL particles are also capable of transporting long noncoding RNAs (lncRNAs), suggesting that lipemia could affect a broader spectrum of circulating noncoding RNAs (ncRNAs) [25]. Despite these potential interactions, the direct influence of lipemia on ncRNA isolation and analysis remains insufficiently explored.

Lipemia is often indicative of underlying metabolic or cardiovascular diseases and presents a particular challenge in clinical studies since it cannot always be effectively controlled through standard 12-hour fasting protocols. Consequently, it is essential to disclose the presence of lipemia in the samples and, whenever feasible, quantify its extent and be aware of differences in circulating miRNAs that may be attributed to differences in lipoprotein levels.

Medication

Certain acute therapies administered in clinical situations, such as during emergency hospitalizations for myocardial infarction or prior to surgical/diagnostic interventions (e.g., coronary angiography, elective percutaneous coronary intervention, percutaneous balloon angioplasty or surgical myocardial revascularization), may influence the circulating miRNA profile. One key example is anticoagulation therapy with heparin, often administered upon admission but before blood sampling. While the necessity of these practices is acknowledged in clinical care, it is important to document and report their use with precision.

Heparin is known to interfere with miRNA quantification, particularly when using qPCR [26,27]. This interference is especially relevant in patients undergoing treatments for conditions such as myocardial infarction, where the timing and dosage of heparin therapy significantly affects blood sample composition. The concentration of heparin in the blood at a given time point depends on several factors, including the dosing schedule and individual pharmacokinetics, which introduces variability in miRNA measurements.

To mitigate the confounding effects of heparin, different heparinase enzymes could be used (either to plasma or RNA) prior to miRNA quantification. Heparinase treatment has been shown to effectively counteract the influence of heparin, thereby enhancing the accuracy of miRNA quantification [28,29]. As such, it is important to acknowledge and report the use of heparin in study subjects, including details on the timing of administration relative to blood sampling, heparin dosage and the method used to neutralize its effects.

Beyond heparin, other commonly prescribed cardiovascular medications may also influence circulating miRNA levels. For example, statin therapy has been shown to modify the expression profile of plasma exosome-derived miRNAs [30]. Likewise, antiplatelet agents such as aspirin affect platelet-derived miRNAs, thereby altering circulating miRNA profiles [31]. Recognizing these potential effects is essential, and future studies should systematically document concomitant pharmacological treatments to account for their possible impact on circulating ncRNA measurements.

Exercise

Existing research underscores the substantial influence of acute or chronic exercise on circulating miRNAs associated with cardiovascular health [32,33]. The analysis by Barber et al. [34] involved 20 previously sedentary adults from the HERITAGE Family Study and measured the expression of 53 miRNAs related to cardiovascular disease in serum collected at baseline and after 20 weeks of endurance exercise training. The study found that regular exercise resulted in significantly decreased mean serum expression of nine miRNAs (let-7b-5p, let-7e-5p, miR-7-5p, miR-25-3p, miR-29b-3p, miR-29c-3p, miR-92a-3p, miR-93-5p and miR-486-5p) and increased mean expression of five miRNAs (miR-27b-3p, miR-126-3p, miR-142-3p, miR-146a-5p and miR-221-3p).

These findings highlight how regular physical activity, even moderate or casual exercise, alters the circulating profile of specific miRNAs. In this context, it becomes recommended to record and disclose all forms of physical activity prior to blood sampling.

Sample Pretreatment

In specific situations, biological samples require pretreatment procedures to ensure safety during handling, particularly when dealing with pathogens that present significant health risks, such as during the recent COVID-19 pandemic. In such cases, a previous step involves the inactivation of infectious agents to mitigate transmission risks without compromising the integrity of RNA for subsequent molecular analyses.

For RNA isolation, reagents containing guanidine salts and phenol are widely employed and serve the dual purpose of inactivating pathogens and preserving RNA integrity. These reagents effectively prevent RNA degradation and maintain the reliability of molecular profiling. However, alternative inactivation protocols have also been proposed, including heat treatment at temperatures up to 70°C and the use of surfactants like Triton X-100.

Despite their potential for safe sample handling, the effects of these alternative inactivation methods on RNA isolation efficiency and downstream detection processes remain unclear. Heating, for example, may affect RNA structure or integrity. Given this uncertainty, it is crucial to carefully document the inactivation method used in sample processing.

Conclusions

The successful translation of circulating miRNAs into reliable clinical biomarkers depends on strict adherence to Good Laboratory Practice (GLP) principles. Following standardized procedures and ensuring meticulous sample handling greatly improves the reliability and reproducibility of results.

To enhance transparency and facilitate reproducibility, it is essential that all relevant information be thoroughly and accurately documented in the Methods section of scientific publications. By adopting and prioritizing these best practices, the scientific community strengthens confidence in the clinical utility of circulating miRNAs and accelerate their integration into diagnostic and prognostic workflows.

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Conflict of interest

YD has filled patents related to the use of miRNAs for diagnostic and therapeutic purposes and is a member of the Scientific Advisory Board of the molecular diagnostic company Firalis SA.

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