TECHNOTE 1 - MINIMUM PREANALYTICAL INFORMATION FOR THE PUBLICATION OF STUDIES ON CIRCULATING CELL-FREE MICRORNA-BASED BIOMARKERS

TechNote 1 - Minimum Pre-analytical Information for the Publication of Studies on Circulating Cell-Free microRNA-based Biomarkers

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1. 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 significant 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. Several factors contribute to this issue, including technical variability in sample collection, storage, processing, RNA isolation methods, miRNA quantification techniques and data analysis tools (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 (3, 4). Establishing 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 accelerate the utilization of multiple omic technologies to bring novel paradigms in prevention, diagnosis and treatment of atherosclerotic cardiovascular disease (6).

2. Minimal Pre-analytical Information

2.1 Timing for Blood Sampling and Processing

The timing of blood sample collection is a key pre-analytical variable typically governed by standardized protocols that require an 8- to 12-hour fasting period in the early morning. This minimizes the impact of postprandial states (e.g., lipemia) and circadian fluctuations. However, timing presents a particular challenge in studies on 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 taken into account. Delays in processing 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). A total of 53 of the 179 miRNAs examined showed significant changes, including miRNAs proposed as endogenous controls (e.g., 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.

2.2 Sample Type

The identification of the sample type is of vital importance to take into account the effects of different anticoagulants and stabilizers on miRNA quantification. A variety of tubes for sample collection are available, including those designed for serum (with clot activator and separation gel) and plasma (EDTA, sodium citrate, heparin, oxalate, etc.). On the market there are also systems intended for purposes of molecular diagnostic testing. BD Vacutainer® PPT™ is a closed system allowing separation and storage of undiluted EDTA plasma in the primary blood collection tube. Additionally, specialized tubes, such as PAXgene® Blood RNA Tube, Streck- RNA Complete BCT® and cf-DNA/cf-RNA Preservative Tubes (Norgen Biotek Corp), aim to preserve RNA integrity.

While an ideal solution would be the use of tubes coated with RNA preservatives, recent assessments by 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 processing 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

results. 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 reported based on the type of anticoagulant used, with variations observed among EDTA, citrate and CTAD (10).

It should be noted that heparin interferes with PCR reactions (11), as this anticoagulant binds to calcium and magnesium ions, components of the PCR master mix. Consequently, when isolating RNA for RT-qPCR, it is advisable to avoid blood collection tubes containing heparin (12). Citrate, on the other hand, may dilute plasma and potentially induce hemolysis.

2.3 Centrifugation Protocol

Accurately describing the conditions used to obtain plasma or serum ensures reproducibility and precision in miRNA research. Protocols for obtaining serum, plasma or platelet-poor plasma (PPP) vary widely among laboratories, particularly 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 undertaken to standardize the centrifugation protocol. 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 protocols when specific miRNA carriers, such as lipoproteins, are being analyzed. Lipoproteins share biophysical properties with exosomes, which makes miRNA profile analysis more challenging (18).

2.4 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 and 30 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.

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.

2.5 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-16-5p, 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 (21). 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.

2.6 Lipemia

Currently, there is a notable lack of studies that thoroughly evaluate the impact of lipemia on circulating miRNAs. Lipoproteins, which play a significant role as miRNA carriers (22), may affect the circulating miRNA profile, especially under conditions of elevated lipoprotein levels, such as the postprandial state. Despite this potential interaction, the direct influence of lipemia, characterized by abnormally high levels of lipids in the blood, on miRNA isolation and analysis remains underexplored.

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 becomes pivotal to disclose the presence of lipemia in the samples and, whenever feasible, quantify its extent.

2.7 Medication

Medication of study subjects is typically well-documented into the inclusion/exclusion criteria during patient recruitment and sample collection. However, 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 still important to document and report their use with precision.

Heparin is known to interfere with miRNA quantification, particularly when using qPCR (23, 24). 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 (25, 26). 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.

2.8 Exercise

Existing research underscores the substantial influence of prolonged exercise on circulating miRNAs associated with cardiovascular health (27). The analysis by Barber et al. (28) 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 is recommended to record and disclose all forms of physical activity prior to blood sampling.

2.9 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 translation of circulating miRNAs into reliable biomarkers requires adherence to Good Laboratory Practice (GLP) principles. Following recommended procedures and ensuring meticulous sample handling significantly enhance the reliability of results.

To promote transparency and facilitate the replication of experiments, it is imperative that the pertinent information is meticulously and accurately documented in the Methods section of scientific publications. By prioritizing these practices, the scientific community could strengthen confidence in the utility of circulating miRNAs as biomarkers for clinical applications.

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Conflict of interest

YD has filed 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.

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).

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