

NCI Biospecimen Evidence-Based Practices		CELL-FREE miRNA: BLOOD COLLECTION AND PROCESSING			
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1. PURPOSE

The purpose of this document is to provide evidence-based guidance for the proper collection and processing of cell-free microRNA (cfmiRNA) from human plasma and serum. This guidance is intended to support the development and execution of evidence-based Standard Operating Procedures (SOPs) for human biospecimen collection, processing, and storage.

2. SCOPE

This evidence-based best practice document is applicable to the collection, processing, isolation, and storage of circulating cfmiRNA from plasma or serum that is intended for analysis in clinical and/or research settings; it does not include the requisite processing for analysis of exosomal miRNA. The ISO 21899:2020 standard (Reference 9.1.9) may be useful as a reference for validation and verification of SOPs produced using this BEBP.

3. DEFINITIONS

- 3.1. Anticoagulant:** A substance that is used to prevent and treat blood clots in blood vessels and the heart; also called blood thinner; any agent capable of preventing blood clot formation
- 3.2. Serum:** The clear liquid portion of the blood that remains after blood cells and clotting proteins have been removed
- 3.3. Plasma:** The clear, yellowish, fluid portion of the blood that carries blood cells; the proteins that form blood clots are in plasma
- 3.4. Aliquot:** A portion of the total amount of the biospecimen collected
- 3.5. cell-free microRNA (cfmiRNA):** microRNA found in the bloodstream and generally measured in serum or plasma
- 3.6. Processing delay:** The time between venipuncture and centrifugation of blood to obtain plasma
- 3.7. Clot time:** The time between venipuncture and centrifugation of blood to obtain serum
- 3.8. Extraction delay:** The time between the completion of plasma or serum processing and RNA extraction
- 3.9. Interim plasma/serum storage:** The duration between the transfer of plasma (or serum) to new tube(s) and analysis

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3.10. Freeze-thaw cycles: The number of times a biospecimen or sample has been frozen and then thawed

4. ENVIRONMENTAL HEALTH & SAFETY

4.1. Universal Precautions (CDC-2007) and guidelines associated with Coronavirus Disease 2019 (COVID-19) and Ebola should be used for all phases of blood collection and processing, and cfmiRNA processing (Reference 9.1.1, 9.1.2 and 9.1.3).

5. RECOMMENDED MATERIALS/EQUIPMENT

5.1. Appropriate safety equipment as described in published guidelines (References 9.1.1, 9.1.2 and 9.1.5).

5.2. Plastic-backed absorbent bench paper

5.3. Blood collection tube of choice (See 7.1 and 7.2)

5.4. Antiseptic wipes

5.5. Vacutainer needle (21-23 gauge) with hub or butterfly needle with Luer adapter

5.6. Tourniquet

5.7. Phlebotomy chair

5.8. Refrigerator (4°C)

5.9. Hi-speed centrifuge

5.10. Falcon tubes

5.11. Storage tubes, such as cryotubes, suitable for centrifugation, storage at -80°C and amenable to waterproof labeling using barcodes or with unique identifiers. RNase-free tubes are suggested.

5.12. LoBind tubes

5.13. Pipettes and sterile RNase-free tips for transfer

5.14. Freezer ($\leq -80^{\circ}\text{C}$) for long-term storage. A -30°C freezer is acceptable if the anticipated duration of frozen storage is ≤ 1 year.

6. PROCEDURAL GUIDELINES

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6.1. Recording of biospecimen pre-acquisition data

6.1.1. Whenever possible, extensive data relating to preacquisition conditions that may affect the integrity of the biospecimen should be recorded. Such data must include patient information (including age, gender, fasting status, diagnosis, physical activity level, and treatment type(s) and date(s)) as well as details relating to biospecimen acquisition (including number of venipuncture attempts, patient position, tourniquet usage, and date and time of blood collection) (Reference 9.1.4). When possible, the complete blood count of the blood specimen should be recorded as it may be informative in identifying potential sources of bias. Appropriate authorization by HIPAA or another pertinent regulatory agency and informed patient consent must be obtained prior to the collection of patient blood and data for research purposes (Reference 9.1.7).

6.1.2. Label each collection tube with unique unambiguous identifiers, such that the tube can be readily matched to all relevant patient and specimen handling data (Reference 9.1.4). Ensure that all labels are robust to all handling steps including but not limited to frozen storage, water, and commonly used solvents. The labeling scheme should accommodate the real-time documentation/recording of pre-analytical conditions (See 6.5.5 for additional details).

6.2. Collection tube considerations

6.2.1. Collection tubes containing ethylenediaminetetraacetic acid (EDTA), sodium citrate, acid citrate dextrose (ACD), citrate-theophylline-adenosine-dipyridamole (CTAD), or sodium-fluoride/ potassium-oxalate (NaF/KOx) are acceptable for blood collection as are select proprietary tubes containing a preservative such as Streck cfDNA BCT, Roche Cell-Free tubes, or Norgen cfDNA/cfRNA tubes)(See 7.3). When specimen processing within 1-2 h of collection is not possible, then use of preservative-containing collection tubes is preferred. Use of serum tubes may also be acceptable if processing is well-controlled (See 7.2 and 8.3.2). Use of heparin, Streck RNA BCT, or PAXgene tubes is not advisable (See 7.3). Optimally, tube choice should be validated during study design and remain consistent throughout the duration of the study. If changes to tube type are necessary, they must be validated in a pilot study as miRNA levels may differ between serum and plasma or among different anticoagulants (See 7.3, and 8.3.2).

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6.2.2. Blood collection tube choice may also be guided by specimen availability and the need to assess multiple analytes (cfmiRNA, cfDNA, etc.) from a single collection tube (See 8.3.1).

6.2.3. To ensure sufficient yield, ideally a minimum of 10 mL of blood per tube should be collected (Reference 9.1.6). However, the total volume of blood required will depend on both the analytical method and the anticipated abundance of the cfmiRNA of interest. Consequently, smaller volumes may be sufficient. Care should be taken to avoid underfilling blood collection tubes containing an anticoagulant or preservative (See 8.3.3).

6.3. Blood Collection

6.3.1. Blood collection after the subject has fasted for > 6 h is preferred, as eating prior to venipuncture may result in a diet-specific increase in fatty acids in plasma/serum (See 8.3.3).

6.3.2. Blood collection tubes should be stored at room temperature (20-24°C) unless otherwise indicated by the manufacturer (See 8.3.2).

6.3.3. The patient must be seated for at least 5 minutes before venipuncture with the arm positioned on a slanting armrest such that there is a straight line from the shoulder to the wrist (Reference 9.1.6).

6.3.4. Apply a tourniquet 3-4 inches above the venipuncture site (Reference 9.1.5) with enough pressure to provide adequate vein visibility. Have the patient form a fist. Select the median, cubital, basilic, or cephalic veins for venipuncture (References 9.1.4 and 9.1.6). Collection from a port should be avoided (References 9.1.5 and 9.1.6). A vein imager should be used when available to improve venipuncture technique.

6.3.5. Clean the venipuncture site with an antiseptic wipe in a circular motion beginning at the insertion site (References 9.1.5 and 9.1.6) and allow to air dry. Once the skin is completely dried, anchor the vein by placing your thumb 2 inches below the site and pulling the skin taut to prevent the vein from moving (References 9.1.5 and 9.1.6).

6.3.6. Insert the 21-23 gauge butterfly needle (See 8.3.3) with Luer adapter into the vein at 30° angle and then push the evacuated tube into the hub or adapter (References 9.1.5 and 9.1.6).

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6.3.7. Once blood flow is established, release the tourniquet (total elapsed tourniquet time should be < 1 min) (References 9.1.5 and 9.1.6) and ask the patient to open their hand.

6.3.8. Make sure that tube additives do not touch the stopper or the end of the needle during venipuncture (Reference 9.1.6).

6.3.9. Non-cfmiRNA clinical specimens should be collected first (See 8.3.3). If no other blood specimens are being collected, discard the first 2-3 mL of blood prior to collecting blood specimens for cfmiRNA analysis (Reference 9.1.6; See 8.3.3).

6.3.10. After completely filling the tube, immediately remove the tube leaving the needle inserted and slowly and gently invert the tube as recommended by the manufacturer for the tube type of choice (Reference 9.1.6).

6.3.11. After filling the last collection tube, place gauze over the puncture site and remove the needle (Reference 9.1.5).

6.3.12. Store anticoagulant and serum tubes containing blood specimens upright (Reference 9.1.6) at room temperature (20-25°C) (See 7.4, 7.5, 8.3.4 and 8.3.5).

6.4. Processing Delay

6.4.1. In most instances, blood specimens should be centrifuged as soon as possible, optimally within 2 h of venipuncture (See 7.4, 7.5, 8.3.4 and 8.3.5). However, if proprietary tubes containing a preservative are used, then a pre-centrifugation delay of 24-48 h at room temperature is acceptable (See 7.3 and 8.3.4). Serum separator tubes should be processed immediately, but serum tubes relying on natural clot formation should be processed after 1 h at room temperature (See 8.3.5).

6.4.2. Regardless of tube type, agitation of blood should be minimized during a processing delay as hemolysis alters cfmiRNA levels (See 7.6).

6.5. Blood Processing

6.5.1. Centrifuge blood collection tubes using a protocol validated for the tube type (See 8.3.6). Acceptable centrifugation speeds and durations include the following ranges, 820-3,500 x g for 1-20 min at 4°C or room temperature (See 7.7).

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6.5.2. If a two-step centrifugation is desired transfer plasma or serum to a new Falcon tube (or an equivalent container), carefully leaving the buffy coat behind.

6.5.3. Further removal of platelets, white blood cells, and cellular debris can be achieved through a second centrifugation at 10,000-16,000 x *g* for 15 min (See 7.8) or filtration (See 7.9 and 8.3.6). While the suitability of including a filtration or second centrifugation step will depend on the abundance of the miRNA of interest, a secondary processing step is generally recommended with the notable exceptions of miRNAs with low levels of expression and discovery-based studies (See 7.9, 7.8 and 8.3.6).

6.5.4. Serum and plasma specimens with evidence of hemolysis must be excluded from cfmiRNA analysis (See 7.6). Hemolysis should be quantified by measurement of hemoglobin concentration or when possible, using the ratio of miR-23a to miR-452 or miR-451a (See 7.6). Use of a spectrophotometer or other visual method is not recommended due to low detection sensitivity (See 7.6). Residual platelet count should be enumerated by flow cytometry or an impedance-based method (See 7.7 and 11.3).

6.5.5. Plasma or serum should be aliquoted into multiple tubes suitable for cryostorage at $\leq -80^{\circ}\text{C}$. Generally, a plasma or serum aliquot of 100-1,000 μL is adequate for a single extraction using a proprietary kit; however, the volume of plasma or serum required will depend on several factors that include extraction method, assay, and anticipated cfmiRNA abundance. Aliquot volume should be chosen to optimize cfmiRNA detection and avoid multiple freeze-thaw cycles. Optimally tubes should be RNase-free. While a barcode labeling system is recommended, any clear and robust labeling system that can withstand frozen storage and common solvents is acceptable (See 8.3.7). The labeling system should accommodate annotation of the preanalytical conditions experienced by each aliquot; please consult the International Society for Biological and Environmental Repositories (ISBER)'s Sample PREanalytical Code (SPREC) as a comprehensive example of biospecimen documentation (Reference 9.1.8).

6.6. Interim Plasma/Serum Storage (Note: not applicable for exosome analysis)

6.6.1. Optimally, cfmiRNA analysis should be conducted immediately, whether after extraction or by direct analysis of plasma or serum (See 7.10, 7.11 and 8.3.7). However, if immediate analysis is not possible, storage of plasma at room temperature or 4°C for up to 3 h, at -20°C for several months, or -80°C

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for years is acceptable for most cfmiRNA endpoints. Potential effects of each storage temperature should be carefully considered for the targeted miRNA(s); for example, effects associated with freeze-thaw cycling may be more severe than a pre-extraction delay at 4°C. Freeze-thaw cycling should be avoided (See 7.12 and 8.3.8). Interim storage of serum should be avoided (See 7.10 and 7.11).

6.6.2. Frozen serum and plasma aliquots should be thawed on wet ice with occasional gentle mixing for the minimum time necessary (See 8.3.8).

6.6.3. Thawed serum and plasma should be thoroughly mixed and centrifuged at 1,000-1,600 x *g* for 1-2 min to pellet cryoglobulins immediately prior to miRNA extraction or analysis (See 7.12 and 8.3.8).

6.7. miRNA Extraction and Quantification

6.7.1. For analytical methods requiring extraction (See 8.3.9), a miRNA-specific commercial extraction kit is preferred, but phenol-chloroform based methods are acceptable (See 7.13). The expert panel advises against the use of column-based extraction kits for miRNA discovery studies (See 8.3.9).

6.7.2. Optimally, analysis should be performed immediately following extraction (See 7.14 and 8.3.10). Based on reports in the literature, interim storage of extracted miRNA or cDNA in low bind tubes at or below -80°C may be acceptable for several months (See 7.14). However, the expert panel advises that long-term storage should be limited to plasma/serum specimens, and if short-term storage of extracted miRNA is required then Tris buffer or ethanol should be used as the medium (See 8.3.10).

6.7.3. cfmiRNA levels quantified by real-time PCR should be expressed as an average quantification cycle (Cq) value relative to the Cq values of two or more constitutively expressed miRNA transcripts that display stable expression across the experimental conditions and disease states anticipated during the study (See 7.15 and 8.3.11). Additionally, miRNA/RNA used in any assay can be quantified using capillary electrophoresis and/or fluorometric methods, but these methods are unacceptable if extraction included carrier RNA.

6.7.4. All analytical methods used should be evaluated for reproducibility and standardized to minimize platform-specific effects (See 7.15 and 8.3.11). The ISO 21899.2020 standard (Reference 9.1.9) may be useful as a reference for further validation and verification.

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7. SUMMARIES OF LITERATURE EVIDENCE

An inclusive approach was applied when composing the Summaries of Literature Evidence section; therefore, all studies that were identified and reviewed are presented and cited when relevant. Critical review of individual studies was limited; for example, larger well-controlled studies may be highlighted within the text, but smaller and/or flawed studies are also discussed. This approach was applied so that individual readers of the BEBP can assess and evaluate the evidence presented and its impact on their particular work.

7.1. Preacquisition:

Variables such as patient age, gender, fasting status, and exercise as well as the time of blood collection may each affect miRNA expression and thus must be carefully recorded and considered in any analysis. Many cfmiRNA levels show extremely high intra-individual variation in both serum and plasma and this variation must be considered in any assay [1, 2]. Patient age and gender are known to influence the levels of some [3, 4] but not all [3-6] miRNAs. The effects of fasting status on cfmiRNA levels are unclear, with some studies reporting significant differences [7-9] while others observed nonsignificant fluctuations [10, 11]. Although evidence is limited, one study reported exercise significantly affected levels of several miRNAs [12], while another reported an interaction between the timing of exercise relative to when the individual last ate [8]. Further complicating analysis, some but not all miRNAs display a rhythmic fluctuation in levels based on the time of day the specimen was sampled [13].

7.2. Serum versus Plasma:

The literature supports use of either serum or plasma for cfmiRNA analysis; however they are not interchangeable specimen types as the levels and stability of individual cfmiRNAs can differ between serum and plasma [6, 14-21](See Tables 10.1 and 10.2). The magnitude of differences between serum and plasma have been attributed in large part to differences in preanalytical handling [22], patient disease state [23], and clot time (See 7.4). In the absence of extensive processing delays, plasma generally displays a greater diversity of miRNA species than serum [15, 16], but clotting time is a confounding factor (See 7.4). Although 94% of the miRNAs identified in serum were also present in plasma/platelet poor plasma, only 35-78% of the miRNAs identified in plasma were present in serum [15, 16]. cfmiRNA from clotted blood samples can be deceiving as PBMCs and platelets broken up during clotting can release miRNA. With a few notable exceptions [19, 20], serum tends to have levels of individual miRNAs that are higher or comparable to those in plasma

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specimens [6, 16-18, 24], including those indicative of hemolysis [17]. Differences between plasma and serum may also include the coefficient of variation and disease state-induced alterations in cfmiRNA levels [23]. Differences in serum and plasma processing may be partially responsible for the reported dissimilarities in miRNA detection. Hemolysis has been shown to increase levels of miR-15b, miR-16 and miR-24 [19]; and centrifugation speed affects the ratio of individual miRNA levels in serum versus plasma [22]. In conclusion, plasma and serum may not be used interchangeably and the optimal specimen type will depend on the cfmiRNA targeted, workflow constraints such as processing delays, and the assay used for quantification.

7.3. Anticoagulant/ Tube Type:

Anticoagulation with K₂EDTA [5, 7, 25-30], sodium citrate [7, 25-27, 29, 30], ACD [25], CTAD [29], NaF [30] and NaF/KOx [26] or cell stabilization tubes by Streck, Norgen, and Roche are all acceptable for cfmiRNA detection analysis; however, heparin should be avoided due to potential interference in enzyme-based molecular assays [7, 20, 25-27]. Notably, anticoagulant-specific differences in individual cfmiRNA levels have been reported. Mean levels of miR-16 and miR-223 were slightly higher when quantified in sodium fluoride (NaF)/potassium oxalate (KOx) plasma than in EDTA or citrate plasma [26]. Further, the addition of NaF/KOx to EDTA plasma resulted in a doubling of miR-16 levels [26]. Although plasma collected in platelet preparation tubes (PPT) had more cfmiRNA species detected by real-time PCR than K₂EDTA plasma [31], differences in cfmiRNA stability may be a confounding factor [31]. Similarly, alpha diversity was found to be significantly lower in specimens anticoagulated with K₂EDTA than ACD, citrate or CTAD [32]. When plasma is destined for cfmiRNA analysis, heparin should be avoided as it interfered with miRNA quantification by PCR [25, 26]. While treating plasma with heparinase rescued miR-16 detection [26], the extent of heparin-mediated enzyme inhibition may depend on the details and conditions of cfmiRNA extraction. Pre-analytical factors may also confound analysis of anticoagulant-specific effects on cfmiRNA levels. For example, compared to citrated plasma, K₃EDTA plasma had higher levels of miR-451a [20, 32] and other hemolysis-associated miRNAs [32] and lower levels of platelet-derived miRNA [32], which may be due to differences in either anticoagulant or the degree of hemolysis. The effects of pre-centrifugation storage on cfmiRNA levels in plasma was dependent on anticoagulant [25].

If processing delays > 6 h are anticipated, collection tubes containing a preservative have been reliably used for miRNA analysis, although the stability of miRNA differs between tube types (See Tables 10.1 and 10.2). Total cfmiRNA levels in plasma

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remained unaffected when blood was stored at room temperature in Streck, Norgen, and Roche cell-free DNA tubes for up to 1 week [28]. However, blood collected in PAXgene Blood tubes displayed an increase in cfmiRNA levels following a pre-centrifugation delay of 24 h at room temperature [28], and blood collected in Streck RNA BCT had significant declines in individual miRNA levels following storage at room temperature for 24 h or more [33]. Blood collection tubes containing preservatives also differed in their ability to prevent contamination introduced by red blood cell (RBC) lysis. Hemolysis was first observed among specimens stored for 6 days in Streck cfDNA tubes or 7 days in Roche Cell-Free DNA Collection Tubes but was not observed in specimens stored in Norgen cfDNA/cfRNA tubes for up to 7 days [28]. Although additional study is required, one report observed less variability in quantified cfmiRNA levels among blood specimens collected in Streck cfDNA tubes compared to those collected in Streck RNA tubes [17].

7.4. Clot time for Serum:

Blood collected into serum separator tubes should be processed immediately (within minutes) and those relying on natural clot formation should be processed within 1 h. Processing delays result in rapid increases in levels of select miRNAs in serum specimens due to hemolysis (See 7.6) and/or high expression in RBCs and platelets (See Table 10.3). Although refrigerated or room temperature storage of blood in SSTMII Advance tubes for up to 9 h did not alter miRNA concentration [25], an increase in hemolysis was observed when blood collected in plain tubes was stored on ice for 2 h [34]. Altered abundance of individual cfmiRNAs assayed by real-time PCR were also observed after 24 h [34] or 4 days [27]. The abundance of miR-21 (or miR-21-5p) increased significantly in some specimens after a clot time of up to 24 h [34] or 4 days [27], while levels of miR-142-3p declined in 2 of 5 specimens that had a clot time of up to 24 h [34]. Importantly, more effects were attributed to the temperature (room temperature versus refrigerated) than the clot time, as less variability in cfmiRNA yield was observed after refrigerated storage than room temperature storage [25].

7.5. Delayed Centrifugation for Plasma Isolation:

When cfmiRNA analysis is anticipated, a room temperature delay to plasma processing by centrifugation should be limited to 2 h or less. Effects observed after a room temperature delay to centrifugation included changes in cfmiRNA yield [25, 28] or cfmiRNA profile in plasma specimens. Storage of EDTA blood at 4°C for > 2 h led to progressive increases in hemolysis [35] and lower library concentrations, a lower percentage of reads mapped, and changes in levels of some miRNAs after 3 days [30] although the window of stability was influenced by tube type and included

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miRNA-specific differences in sensitivity [29, 36]. Effects attributable to a centrifugation delay were anticoagulant- and preservative-specific as total cfmiRNA yield was altered beginning after a delay of 9 h when specimens were stored in tubes containing EDTA or ACD, although no such effects were observed with citrate tubes [25]. Among tubes containing preservatives, miRNA yield was affected beginning after a 24 h delay to centrifugation for PAXgene tubes [28] while no such effects were observed among Streck, Roche, or Norgen cfDNA tubes when specimens were stored for up to 1 week [28]. Individual studies reported different windows of stability even when the same anticoagulant was used; for example, altered cfmiRNA profiles in K₂EDTA plasma were reported beginning after a centrifugation delay of 2 h [34, 37], 3 h [38], 12 h [36] or 72 h [27] compared to those processed with 30 min. Although principal component analysis (PCA) distinguished plasma specimens stored for 24 h at room temperature before centrifugation from those stored for shorter durations based on the cfmiRNA transcriptome, specimens stored for 24 h at 4°C before centrifugation co-clustered with those stored for shorter durations [39]. Notably, several miRNAs remained stable after a centrifugation delay of 24 h [34] or 72 h [33, 36]. Altered levels of individual miRNAs were first reported after an 18 h delay for plasma collected in PAXgene tubes [40], and after 24 h for plasma collected in Streck cfDNA BCT tubes [33]. Confounding analysis, many storage-associated effects are miRNA-specific and detectability of changes will depend on expression and the analytical platform sensitivity. The following miRNAs are reported to be stable after a delay of ≥ 24 h at 4°C when compared to < 1 h: miR-21 [36], miR-27a [36], miR-142-3p [36], miR-218 [36], miR-374-5p [36], miR-376c [36], miR-485-3p [36], miR-520d-5p [36], miR-523 [36]. The following cfmiRNAs were shown to be stable after a delay of ≥ 12 h at room temperature compared to < 1 h: miR-1 [38], miR-16 [41], miR-23 [38], miR-223 [41] and miR-423-5p [38]. Levels of cfmiRNAs expressed in blood cells (See Table 10.3) may increase rapidly and concomitantly during a delay to centrifugation, as hemolysis increases with K₂EDTA blood storage [17, 28, 38] (See 7.6). The window of stability for some cfmiRNAs may be extended if hemolyzed specimens are excluded from further analysis, or if the cfmiRNA of interest is not present in blood cells. Further confounding analysis, windows of stability can differ between cohorts within a study, as an effect of room temperature storage in K₂EDTA tubes was observed in one cohort after 3 days while no effect was observed in a second cohort after 4 days of storage [27].

7.6. Hemolysis:

Specimens with visual evidence of hemolysis should be excluded from analysis and the addition of a real-time PCR based assay for hemolysis is recommended.

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Hemolysis was associated with increased levels of RBC-specific miRNAs in serum [19] and plasma [42-44] (See Table 10.3 for a list of affected miRNAs) and a decrease in the number of miRNAs detected at a given read depth [45], while other miRNAs remained unaffected [19, 42-44]. Interestingly, a single study found hemolysis may have a stabilizing effect on select cfmiRNAs in serum [46]. Importantly, the cfmiRNAs detected in hemolyzed and non-hemolyzed serum were different, with 231 miRNA having a difference of 3-fold or greater when case-matched severely hemolyzed and non-hemolyzed specimens were compared [10]. Imposing cut-off values of < 0.2 absorbance units at 414 nm has been shown to effectively eliminate RBC-contaminated specimens and hemolysis-associated miRNAs artifacts [17, 44]. Alternatively, a threshold difference of five or eight cycles for miR-23a and miR-452 has been successfully used to identify a moderate or severe risk of hemolysis, respectively [47]; while, a threshold difference > 8 cycles for miR-23a-3p and miR-451a successfully detected hemolyzed specimens [17].

7.7. Initial Centrifugation:

Centrifugation speed affects both the number of miRNAs detected as well as levels of individual cfmiRNAs; speeds of 820-3,500 x g for 1-20 min are considered acceptable for cfmiRNA analysis of plasma/serum. Faster centrifugation speeds led to the detection of fewer cfmiRNAs [14, 19, 48], as well as reduced levels of some but not all cfmiRNAs [17, 19, 48, 49], although the magnitude of decline (range: 0-8 cycles) was dependent upon the cfmiRNA species [17]. Collectively, evidence supports that effects of centrifugation speed are rooted in cellular contamination. Given that the cfmiRNAs most affected by centrifugation speed include those commonly detected in platelets (See Table 10.3)[14, 48, 50] and/or RBCs (See Table 10.3)[19], centrifugation induced cell lysis is a plausible source of the increase. Further, platelet-rich plasma obtained by a single centrifugation at 600 x g had 1.17-fold more detectable miRNAs than standard plasma obtained by centrifugation at 3,400 x g (325 versus 277 miRNAs)[14]. While only four miRNAs were investigated, a faster centrifugation speed (10,000 x g versus 1,900 x g) led to fewer differences between serum and plasma specimens [22]. The literature supports centrifugation of blood at 820-3,000 x g [2, 7, 16-18, 20, 23, 25, 28, 31, 33, 37, 38, 41, 48-64] to obtain plasma and centrifugation at 1,500-3,500 x g [16-18, 20, 23, 34, 56, 65-70] to obtain serum. While duration is wed to speed, the majority of the studies surveyed centrifuged specimens for 10-20 min to obtain either serum or plasma for cfmiRNA analysis [2, 7, 16-18, 20, 23, 25, 28, 31, 33, 34, 37, 38, 41, 48-50, 54-70], but centrifugation for 30 min at < 2,000 x g has also been reported [52, 53]. Experimental comparisons of centrifugation temperatures are lacking, however centrifugation at 4°C [2, 7, 16, 20,

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23, 38, 41, 48, 52, 54, 59, 65, 67], 20°C [66], and at room temperature [31, 63] have been reported in the literature for subsequent cfmiRNA analysis.

7.8. Second Centrifugation:

A second centrifugation step at 10,000-16,000 x g for 15 min is acceptable for the adequate removal of platelets from plasma/serum, although loss of low abundance cfmiRNA is an associated risk. A second centrifugation step has been shown to decrease the number of cfmiRNAs detected [62] and levels of individual cfmiRNAs [17, 19, 25, 60]. As centrifugation is used to remove contaminating platelets and cells, costs and benefits of a second centrifugation step may be dependent on centrifugation speeds. Levels of several individual cfmiRNA were lower among plasma specimens centrifuged at 14,400-16,000 x g after initial centrifugation at 795-2,500 x g [17, 19, 25, 62], but not among specimens centrifuged at slower speeds (3400 x g after 1,900 x g) [14]. Further, while increasing the speed of the second centrifugation from 1,000 to 2,000 x g had little effect on the cfmiRNAs detected [48], fewer platelet cfmiRNAs were detected when the speed was increased to 10,000 x g [48] and less rRNA contamination was observed after centrifugation at 16,000 x g compared to 3,500 x g [71]. Notably, any effects attributable to platelet contamination will compound during storage, as a smaller percentage of cfmiRNAs were affected by storage of platelet poor plasma compared to standard K₂EDTA plasma [31]. Importantly, a third centrifugation step had little [19] or no [72] effect on miRNA abundance in plasma [19, 72] and serum [19]. Notably, effects associated with centrifugation workflow are cfmiRNA transcript-specific. A study investigating the effects of two centrifugations at 3,000 x g for 15 min versus a single centrifugation at 3,000 x g for 30 min observed higher cfmiR-126 detection but no change in the three other cfmiRNAs investigated after two-step centrifugation; however, significance was dependent on the method of miRNA normalization [60].

7.9. Filtration:

Filtration can be an effective alternative to a second centrifugation step for reducing platelet and cellular contamination. The abundance of several miRNAs, particularly those highly expressed in RBCs or platelets (See Table 10.3), was altered when plasma [14, 22] or serum [14] was passed through a 0.1 or 0.22 µm filter. However, filter size should be carefully considered, as levels of the hemolysis marker miRNA-451 were non-significantly lower when one-step centrifugation plasma was passed through a 0.1 or 0.2 µm filter, but higher when passed through a 1 µm filter [22].

7.10. Extraction Delay ≥ 4°C:

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Storage of plasma at 4°C prior to miRNA extraction should be limited to < 3 h. The number of cfmiRNAs identified [31] and levels of individual cfmiRNA in plasma and serum were influenced by the temperature [19, 30, 31, 73] and duration [19, 30, 46] of a delay to miRNA extraction. However, numerous miRNA-specific effects [19, 27, 46, 65, 69, 74] confounded the identification of acceptable durations and temperatures of a delay to extraction. While a 24 h delay to extraction at either room temperature or 4°C led to an increase in the number of detectable miRNAs in EDTA plasma, the same delay led to a decline in detectable cfmiRNAs in PPT plasma and EDTA plasma centrifuged a second time to produce platelet poor EDTA plasma [31]. Acceptable delays will ultimately depend upon the initial level of the cfmiRNAs of interest, the specificity of the assay used for quantification, and the magnitude of changes that are anticipated due to the pathology of the patient. Significant changes in individual cfmiRNAs were reported in plasma specimens following an extraction delay of 24 h at room temperature [27, 31] or 2 weeks at 4°C [74], and in serum specimens after an extraction delay of 1 h [69] or 3 h [46] at room temperature or 6 h at 4°C [65]. A single study investigating post-processing storage of serum at 37°C observed no effect on miR-25, miR-221, or miR-222 levels after 3 h [73]. Compared to a room temperature delay, fewer miRNAs were affected when a 24 h delay to extraction occurred at 4°C [31], although another study reported larger average changes in Cq values when a ≤ 2 h delay occurred at 4°C [37]. The presence of platelets was identified as a confounding factor when exploring effects of a delay to extraction, as platelets store and release miRNA the magnitude of delay-induced changes were smaller in platelet poor plasma than standard plasma [31, 64].

7.11. Extraction Delay \leq -20°C:

The majority of miRNAs evaluated remain stable in plasma and serum when stored at -20°C for several months, or -80°C for several years. Nevertheless, frozen storage of plasma and serum specimens prior to extraction altered some [11, 30, 64, 73-75] but not all cfmiRNAs evaluated [30, 64, 73, 74, 76, 77]; and, the magnitude of change was influenced by storage temperature [78]. While levels of several cfmiRNAs differed between serum specimens stored at -20°C and those that were stored at -80°C for 20 months [78], the majority of cfmiRNAs evaluated had comparable levels [78]. Storage of plasma for up to 30 days at -20°C led to increases/decreases in ≤ 1 miRNA compared to specimens stored for 1 h [30] and all sixteen miRNAs evaluated were unaffected by storage for 12 months at -80°C [77]. The duration of frozen storage is also an important preanalytical factor as the number of cfmiRNAs detected [48] and levels of cfmiRNAs [74, 75] are altered over the course of storage. Effects attributable to the duration of pre-extraction frozen storage include fewer detectable cfmiRNAs (177 versus 202) when K₂EDTA plasma

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was stored at -80°C for > 12 y compared to < 1 y [48], and altered levels of a small number of individual cfmiRNAs when K₂EDTA plasma was stored at -80°C beginning after 1 month [75] or 2 months [74]. Nevertheless, only 1 miRNA was found to differ by > 2-fold between K₂EDTA plasma stored for 3 versus 5 years at -80°C [30]. Similar effects were observed when plasma was stored at -20°C, as declines in both individual miRNA levels and total miRNA levels were observed beginning after 1 month [75] or 5 years [74]. Unfortunately, reports evaluating serum conflict, with some cfmiRNAs reported stable at -20°C for 72 h [69] or 7 days [79] and others affected following -20°C storage for 72 h [19] or 6 y [78]. Potentially confounding variables in the identification of acceptable temperatures and durations of frozen storage include cfmiRNA-specific effects [64, 73, 74]; the presence of residual platelets, RBCs, and white blood cells [50, 64]; and post-thaw mixing [20].

7.12. Thawing and Freeze-Thaw Cycling:

Given the literature conflicts as to the sensitivity of cfmiRNAs in plasma and serum to freeze-thaw cycling, as well as the magnitude and direction of changes when observed, multiple freeze-thaw events should be avoided. Several studies examining either serum or plasma reported increases [78, 80, 81], decreases [74, 79], or no effect [24, 64, 73, 82, 83] on cfmiRNA endpoints following multiple freeze-thaw events. Results conflict even when the same miRNA target is quantified in specimens collected using the same anticoagulant with an overlapping number of freeze-thaw events. For example, in EDTA plasma specimens miR-16 remained stable for up to 8 freeze-thaw cycles in one study [24], but levels were reduced after ≥ 3 freeze-thaw cycles in another [79]. In serum, miR-16 levels remained unchanged in specimens that experienced 2 [82] or 4 [83] freeze-thaw events compared to controls that were thawed once [82, 83], although effects of freeze-thaw cycling will depend upon the miRNAs targeted, their physiological level, and the detection assay used. Potential factors that may have confounded investigations of freeze-thaw cycling on cfmiRNA endpoints include the presence of precipitates in thawed specimens, whether thawed specimens were mixed prior to extraction [20], temperature during thaw [80], the presence of platelets [50], and the anticoagulant used during blood collection [50]. In K₂EDTA and citrate plasma, miR-146a-5p and miR-382-5p levels were altered when the precipitate observed in thawed plasma was removed compared to specimens that were thoroughly mixed; however, no such differences were observed between separated and thoroughly mixed serum specimens [20]. Some effects of freeze-thaw cycling are influenced by anticoagulant and the presence of platelets, as effects of a single freeze-thaw event before versus after a second centrifugation differed among EDTA and citrate plasma [50]. A single study reports that sensitivity to thaw temperature was cfmiRNA-specific in serum; when

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serum specimens thawed on wet ice were compared to those thawed at 37°C, differences ranged from severe (miR-93-5p, miR451a) to minute or absent (miR-21-5p, miR-23-3p) [80].

7.13. miRNA Extraction:

Sufficient cfmiRNA yields (as determined by real-time PCR) for downstream analysis were reported using a wide variety of specialty extraction kits as well as an in-house phenol-chloroform based method. Inclusion of an extraction step led to lower Cq values in TaqMan assays [56], although direct analysis of plasma has been successfully employed for next-generation sequencing (NGS) [84, 85], as well as for real-time PCR when samples underwent purification after reverse transcription [86] or when serum was treated with Tween-20 [87]. Tween can be used to dissolve circulating blood exosomes which often contain miRNA cargo [87]. However, extraction was found to be the source of the majority of inter-assay variability [9]. Proprietary kits for miRNA extraction from plasma recommended in the literature surveyed include miRCURY [55, 70, 88], QiAamp CNA [48], miRNeasy [5, 9, 36, 48, 52, 55, 89, 90], mirVana PARIS [19, 75, 91], Norgen miRNA purification kit [17], NucleoSpin [56], EasyPure [7] and RNAdvance [89]. Phenol chloroform-based extraction methods [18, 20] and guanidium thiocyanate and octanoic acid (Gu/OcA) extractions have also been recommended. Reported effects attributable to extraction kit choice included altered between-sample variance in Cq values [17-19, 92], and differences in NGS-generated cfmiRNA profiles of plasma specimens extracted with different proprietary kits (although differences were smaller than those introduced by library preparation) [93]. The studies that investigated potential effects of miRNA extraction method on serum specimens reported successful cfmiRNA analysis after extraction with the following proprietary kits: miRNeasy [19, 94], mirVana PARIS [19], Trizol-LS [19], Qiazol in combination with dr. GentLE, precipitation [94], Norgen Total RNA isolation kit [94], and miRCURY RNA isolation kit -Biofluids [94]; and while differences between kits were identified [19], the optimal choice was dependent on the miRNA evaluated [94] and post-extraction storage [19]. When serum-derived miRNA samples were analyzed immediately, extraction with miRNeasy or miRVANA kits led to lower Cq variance compared to Trizol LS; but, when miRNA samples were stored for 6 months at -20°C, extraction with Trizol LS led to lower Cq variance than the mirVana PARIS kit [19]. Additional confounding factors that affected evaluation of extraction techniques included the use of carrier RNA such as MS2 [52, 92] or yeast [52, 91] and preheating steps [73]. When MS2 and glycogen were used in combination as an RNA carrier, higher cfmiRNA yields [34] and a lower standard error of the mean were observed than when no carrier was used [34], but excessive amounts of carrier RNA altered

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cfmiRNA profiles [91]. While preheating serum to 75°C for 5 min before RNA extraction lowered mean Cq values [73], incubation at 60°C for 2 h was detrimental.

7.14. Post-Extraction Storage:

Given the need for high powered studies on stability, the conservative recommendation is that frozen storage of isolated cfmiRNA and cDNA samples should be limited to $\leq -80^{\circ}\text{C}$ for the shortest duration required. In one study, frozen storage of extracted miRNA specimens from the blood of *one* volunteer resulted in a decline in the level of miR-451a, but not miR-155-5p, after ≥ 1 day at -80°C [20] and non-significant changes in miR-155-5p and miR-451a after storage of cDNA at -20°C for up to 120 days [20]. Conversely, in a single study the one miRNA investigated (miR-16-5p) was reported to be stable at room temperature for up to 3 days post-thaw in the three specimens examined, but after 7 days a small overall increase along with increased variability was found [64]. Supporting instability of extracted miRNA and cDNA, levels of four mouse cell line miRNAs decreased significantly after only 3 days at -80°C [95]. Importantly, for cfmiRNAs with low expression even a small decrease may lead to loss of detection.

7.15. Quantification:

The method used for miRNA normalization greatly affects the miRNA profile and should be carefully selected; acceptable strategies include normalization to input amount and two or more constitutively and stably expressed miRNA transcripts. General guidance on the adequate validation of a real-time PCR assay can be found in de Gonzalo-Calvo (2022)[96]. Generally, low coefficient of variation (CV)s and a low median CV value were associated with normalization to the global mean Cq [63, 97], quantile [97] or normalization to the three [16] or eight [63] most stable cfmiRNAs in a dataset; however, one study concluded that normalization via geNorm or NormFinder was superior to normalization to the global mean [51]. Others have reported that normalization to a single miRNA increased sensitivity [63] or resulted in a lower CV [16]. Importantly, the best miRNA for normalization may depend on a specific dataset and the miRNA(s) of interest [20]. One study suggests that assays for cfmiRNA in plasma should have a PCR efficiency of $100\pm 10\%$, an intra- and inter-day precision of $< 25\%$ and $< 35\%$, respectively, and a calibration curve with an $R^2 \geq 0.98$ [20].

8. EXPERT-VETTING

8.1. Details of Expert Review:

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Three experts were identified and invited to review the document based on their contributions to the literature regarding the study of cfmiRNAs (See 8.2). Feedback from participants was collected and documented following initial review of the draft BEBP. Final thoughts and recommendations were captured from the expert panel during a scheduled teleconference after review of the BEBP document. Participating individuals did so voluntarily and without compensation.

8.2. Participating Experts:

- ❖ Kevin Elias, M.D.
Director, Gynecologic Oncology Laboratory
Brigham And Women's Hospital
Boston, Massachusetts
kelias@bwh.harvard.edu
- ❖ Dave Hoon, M.Sc, Ph.D.
Director, Depts of Translational Molecular Medicine & Sequencing Center
Saint Johns' Cancer Institute, Providence Health and Service
Santa Monica, California
Dave.hoon@providence.org
- ❖ Tina Lockwood, Ph.D., DABCC, DABMGG
Director, Genetics and Solid Tumor Diagnostics Laboratory
University of Washington
Seattle, Washington
tinalock@uw.edu

8.3. Expert Recommendations

8.3.1. General Considerations:

The expert panel agreed that effects of pre-analytical steps on the quantifiable levels of miRNAs will be transcript-specific. Thus, the panel advised that the complete workflow (from collection to analysis) be validated for each miRNA of interest. Further, the workflow should remain consistent for a given study and any necessary changes to the protocol require validation. Additionally, if differences in workflow and protocols are necessary and/or anticipated (such as due to different collection sites or use of retrospectively collected specimens), then it is crucial to assess their impacts. The panel advised that differentially processed specimens be run and analyzed together to verify that they fall within an acceptable reference range and have acceptable measures of integrity.

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In consideration of the donating patient and the low volume of blood that may be available for research purposes after clinical chemistry, the panel recommended designing collection and processing workflows such that multiple analytes can be assessed from a single blood puncture and blood collection tube.

8.3.2. Blood Tube Type:

While the expert panel agreed that a variety of blood collection tubes are suitable for cfmiRNA detection and quantification, they cautioned that optimal processing and extraction protocols will depend on the tube type selected. Experts agreed that plasma is the optimal biospecimen type for miRNA analysis. They advised that while serum specimens are not preferred for cfmiRNA analysis, data from serum may still be informative with proper validation. Of note, all FDA approved cfDNA assays specify that plasma be used for analysis. The expert panel emphasized that collection and processing protocols (including collection tube type) must remain constant for the duration of the study. If, for example, a change in tube type is unavoidable then the change must be carefully validated, which could include re-running specimens collected in both types of blood collection tubes simultaneously to verify that the resultant data fall within the same reference range and have similar measures of integrity. The expert panel emphasized that serum and plasma specimens are not interchangeable. All experts agreed that blood collection tubes should not be pre-chilled prior to collection, noting that pre-chilling tubes is not common phlebotomy practice and doing so would result in additional and unnecessary variability. They also noted that chilling tubes containing preservatives is against manufacturer recommendations and could result in detrimental effects.

8.3.3. Blood Collection:

The expert panel agreed that blood should be collected with a 21-23 gauge needle, and that needles with a higher gauge (25 gauge) should be avoided given an increased risk of hemolysis. The experts agreed that the first tube of blood collected from a patient should not be used for cfmiRNA analysis due to potential contamination from the skin plug. Clinical chemistry analysis of the first collection tube is acceptable and routine practice; however, if cfmiRNA is the only analyte investigated then the first tube of blood should be discarded. One expert specified that all alcohol should be allowed to evaporate completely from the skin prior to venipuncture to reduce the likelihood of alcohol interference. Another expert advised that blood for cfmiRNA analysis should be collected from fasting patients (> 6 h prior to collection), as eating immediately prior to venipuncture can increase fatty acids in collected plasma/serum. It is also important that the

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tube be filled to the proper volume to ensure the correct anticoagulant/preservative concentration. Under- or over- filling of the tube may influence subsequent downstream analysis.

8.3.4. Processing Delay of Blood for Plasma:

The expert panel agreed that in the absence of stabilizers and preservatives plasma should be isolated within 2 h of blood collection. However, specimens stored for longer durations (up to 18-24 h) may still contain stable cfmiRNAs and provide informative data, but modest changes in some cfmiRNAs are likely due to platelet contamination. In instances when long processing delays (> 2 h) are anticipated, such as when blood specimens will be sent to a centralized laboratory for processing, experts advised that preservative tubes should be used, which will extend the acceptable processing delay to 48 h.

8.3.5. Processing Delay of Blood for Serum:

The expert panel agreed that plasma is preferred over serum for cfmiRNA analysis. Two of the three experts noted a lack of control in the release of miRNA from the clot when serum is used. When cfmiRNA analysis of serum is necessary, one expert advised that specimens be placed on ice and processed immediately if serum separator tubes are used but stored for 1 h (but not > 2 h) at room temperature if a natural clot formation is needed.

8.3.6. Blood Processing:

The expert panel noted that each processing step results in miRNA loss, and that sequential and/or high-speed centrifugation also shears nucleic acids within the sample. Thus, the number and conditions (speed, temperature, duration) of centrifugation and filtration will depend on the abundance of the miRNA of interest. For example, the experts advised limiting centrifugation to a single spin for cfmiRNAs with low abundance. The panel noted that it is essential that the number and conditions of centrifugations be standardized and remain consistent within a study, and that processing workflow be tailored to the properties of the cfmiRNAs of interest during study design. While two experts have not explored filtration due to concerns with loss of small or low abundance cfmiRNAs, one expert found filtration prior to freezing beneficial in the removal of platelets, cells, and cellular debris.

8.3.7. Plasma/Serum Storage:

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The expert panel recommended that under ideal circumstances plasma or serum should be aliquoted and stored in barcoded low bind RNase-free cryotubes (or a comparable tube) at -80°C or colder. An expert noted that the effects of storage are dependent on the amount of platelet and cellular contamination in the plasma. Use of other labeling systems are also acceptable provided labels are unambiguous, permanent, and waterproof. One expert noted that while use of RNase-free tubes is optimal, it is not necessary in all cases. However, vials should be sterile at best.

8.3.8. Thawing of Plasma:

All experts agreed that plasma aliquots should be thawed on ice with gentle and occasional swirling to ensure rapid thawing. Each of the experts noted that immediately after thawing, aliquots are centrifuged at low-speed (between 1,000-1,600 x g) for 1-2 min to pellet any cryoglobulins that may have precipitated during freezing. Multiple freeze-thaw cycles should be avoided. If non soluble particles are observed in the specimen after thawing, it should not be used.

8.3.9. Extraction:

The expert panel agreed that the need for cfmiRNA extraction is dependent on the platform used for analysis. Extraction steps can lead to loss of detection of low level or small miRNAs, and different extraction methods (such as column-based extraction) can potentially introduce bias. As extraction kits will affect each miRNA differently, it is important that the extraction method remains consistent within a study and that any necessary changes to methodology be experimentally validated. Screening studies by directly assaying a panel of miRNA without extraction is preferable if cfmiRNA abundance is low.

8.3.10. Storage of Extracted cfmiRNA:

The expert panel unanimously agreed that extracted cfmiRNA is very labile and recommends analyzing miRNA samples immediately after extraction. The expert panel recommended plasma aliquots be used for long-term storage at -80°C, as opposed to extracted cfmiRNA samples. Short-term storage of extracted cfmiRNA at -80°C is acceptable, when necessary, although the acceptable duration of short-term miRNA storage is dependent on the buffer used. One expert noted that miRNA stored in Tris buffer will be stable for several months, and miRNA stored in ethanol will be stable for up to a year at -80°C. It is advisable to validate the effects of storage on each targeted cfmiRNA panel.

8.3.11. Quality Assessment/Validation:

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The expert panel was in agreement that reporting and quality standards are necessary for cfmiRNA endpoints. The standards specified by the expert panel include (i) the quantity of total cfmiRNA extracted to assess whether recovery is acceptable and to mitigate false negatives due to loss; (ii) evaluation of hemolysis markers as indicators of sample quality; and (iii) for real-time RT-PCR based analysis, identification of two or more reference miRNAs that have demonstrated stability across both the collection/processing workflow and patient disease state(s) that can be used for normalization, as well as to define an acceptable level of variability. One expert suggested defining a stable amount of variation as within 0.5 of a Cq value. For NGS, it is critical to include counts of both positive and negative controls.

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10. Figures and Tables

10.1. Blood collection tube-specific recommendations for plasma

TUBE TYPE	PRECENTRIFUGATION DELAY	CENTRIFUGATION	SHORT-TERM STORAGE	LONG-TERM STORAGE	FREEZE-THAW CYCLING
EDTA TUBE	< 1 h at RT (storage at 4°C may decrease stability)	2 steps required, Second step must be $\geq 10,000\ g$	< 24 h at RT or 4°C	< 30 days at -20, -70 or -80°C	< 1 cycle, Post-thaw mixing critical
SODIUM CITRATE	Limited evidence suggests < 9 h at RT	2 steps	Insufficient literature evidence	Insufficient literature evidence	More stable than EDTA plasma, Post-thaw mixing critical
ACD	$\leq 3\ h$ at RT	2 steps	No reported effects of 24 h at RT	Insufficient literature evidence	No reported effects ≤ 6 cycles
PPT	Insufficient literature evidence	Insufficient literature evidence	Small differences observed when stored for 24 h at 4°C or RT	Insufficient literature evidence	Insufficient literature evidence
STRECK cfDNA BCT	< 24 h, but other reports extend stability	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence
ROCHE	$\leq 5\ days$	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence
NORGEN	$\leq 5\ days$	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence
PAXGENE	< 24 h	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence

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10.2. Blood collection tube-specific recommendations for serum

TUBE TYPE	PRECENTRIFUGATION DELAY	CENTRIFUGATION	SHORT-TERM STORAGE	LONG-TERM STORAGE	FREEZE-THAW CYCLING
SERUM GEL TUBE	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Insufficient literature evidence	Post-thaw mixing is not critical
SERUM SEPARATOR TUBE	< 3 h at RT or 4°C	2 steps	< 24 h at RT or 4°C	< 24 h at -20°C	Insufficient literature evidence
SERUM CLOT ACTIVATOR TUBE	Insufficient literature evidence	Insufficient literature evidence	< 1 h at RT, ≤ 72 h at 4°C	< 72 h at -20°C or -80°C	≤ 4 cycles
PLAIN SERUM TUBE	< 2 h	Insufficient literature evidence	< 6 h at 4°C	Insufficient literature evidence	< 2 cycles, temperature of thaw is a confounding factor
SERUM UNSPECIFIED TUBE	Insufficient literature evidence	Insufficient literature evidence	≤ 3 h at 4°C, ≤ 24 h at RT	< 20 months at -20°C or -80°C	< 10 cycles

RT, room temperature

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10.3. miRNA species highly expressed in red blood cells (RBC) or platelets and their reported sensitivity to hemolysis.

miRNA	RBC expression	Platelet expression	Sensitivity to hemolysis
<i>let-7a</i>	[42]	[14, 15, 42, 98]	[42]
<i>let-7b</i>			[42]
<i>let-7c</i>		[98]	[42]
<i>let-7d</i>		[15]	[42]
<i>let-7e</i>		[15]	[42]
<i>let-7f</i>	[42]	[42, 99]	[42]
<i>let-7g</i>		[15]	[42]
<i>let-7i</i>		[98]	[42]
<i>miR-103</i>	[42]	[15, 42, 98, 100]	[42]
<i>miR-106a</i>	[42]	[15, 42, 98, 100, 101]	[42]
<i>miR-106b</i>			[42]
<i>miR-107</i>	[42]	[42, 98]	[42]
<i>miR-125a</i>		[15]	
<i>miR-1255B</i>	[43]		[43]
<i>miR-126</i>	[42]	[15, 42, 98, 99, 101]	[42]
<i>miR-126-3p</i>	[29]		
<i>miR-127-3p</i>		[15]	
<i>miR-130a</i>		[98]	
<i>miR-130b</i>		[100]	
<i>miR-139-5p</i>		[15]	
<i>miR-141</i>		[14]	
<i>miR-142-3p</i>	[42]	[14, 15, 42, 98, 99, 101]	[42]
<i>miR-142-5p</i>		[98, 99]	
<i>miR-145</i>		[15]	
<i>miR-146a</i>		[15, 98, 101]	
<i>miR-146b</i>		[15]	

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<i>miR-150</i>		[15]	
<i>miR-150-5p</i>	[29]		
<i>miR-151-3p</i>		[101]	
<i>miR-151-5p</i>	[42]	[42]	[42]
<i>miR-15b</i>	[19, 42, 43]	[15, 42, 98]	[19, 42, 43]
<i>miR-16</i>	[42-44]	[14, 15, 42, 99-101]	[19, 42-44]
<i>miR-17</i>	[42]	[15, 42, 100, 101]	[42]
<i>miR-185</i>		[98]	
<i>miR-186</i>		[15, 101]	
<i>miR-19a</i>	[42]		[42]
<i>miR-19b</i>	[42]		[42]
<i>miR-191</i>		[15, 98, 99, 101]	
<i>miR-191-5p</i>	[29]		[66]
<i>miR-197</i>	[29]	[15]	
<i>miR-199a-3p</i>		[101]	
<i>miR-199a</i>		[15]	
<i>miR-19a</i>		[15, 42, 98]	
<i>miR-19b</i>		[15, 42]	
<i>miR-20a</i>		[15, 98, 101]	
<i>miR-20a-5p</i>			[66]
<i>miR-20b</i>	[42]	[15]	[42]
<i>miR-21</i>	[42]	[15, 42, 98, 99]	[42]
<i>miR-21-5p</i>	[29]		
<i>miR-210</i>		[14]	
<i>miR-22</i>		[99]	
<i>miR-221</i>		[15, 42]	
<i>miR-222</i>		[15, 101]	
<i>miR-223</i>	[29, 42]	[14, 15, 42, 98, 99, 101]	[42]
<i>miR-23a</i>		[98, 99]	

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<i>miR-23a-3p</i>	[29]		
<i>miR-23b</i>		[98-100]	
<i>miR-24</i>	[42]	[15, 42, 99-101]	[19, 42]
<i>miR-24-3p</i>	[29]		
<i>miR-26a</i>		[15, 98-101]	
<i>miR-26b</i>		[15, 99]	
<i>miR-27-3p</i>	[29]		
<i>miR-28-3p</i>	[29]	[15]	
<i>miR-30b</i>	[42]	[15, 42, 98, 101]	[42]
<i>miR-30b-5p</i>			[66]
<i>miR-30c</i>	[42]	[15, 42, 98, 101]	[42]
<i>miR-30d</i>		[42]	
<i>miR-30e</i>	[42]	[15, 98]	[42]
<i>miR-320a</i>	[29]		
<i>miR-328</i>		[15]	
<i>miR-331-3p</i>		[15]	
<i>miR-342-3p</i>		[15]	
<i>miR-374a</i>		[15]	
<i>miR-374b</i>		[15]	
<i>miR-383</i>		[15]	
<i>miR-423-5p</i>		[15]	
<i>miR-451</i>	[42-44]		[42-44]
<i>miR-451a</i>	[29]		[37]
<i>miR-484</i>		[101]	
<i>miR-486-3p</i>	[43]		[43]
<i>miR-486-5p</i>	[42]		[42]
<i>miR-486b</i>	[42]		[42]
<i>miR-532-3p</i>	[43]		[43]
<i>miR-574-3p</i>		[15]	

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<i>miR-636</i>	[43]		[43]
<i>miR-652</i>		[15]	
<i>miR-720</i>		[99]	
<i>miR-744</i>		[15]	
<i>miR-886-5p</i>	[43]		[43]
<i>miR-92a</i>	[42-44]	[14, 15, 42]	[42-44]
<i>miR-93</i>	[42]	[15, 42]	[42]

11. REVISION HISTORY

11.1. Revision 1.0 (2/5/2025): 508 Compliance

To achieve 508 compliance, formatting changes were applied, tables were edited, and alternative text was added to the original version (5/1/2023).