

Title: Quality controls and best practices for analyzing microRNAs in cell-free biofluids by RT-qPCR.

Scope

MicroRNAs in liquid biopsies hold great promise as minimally invasive diagnostic biomarkers for a wide range of diseases and biological processes (Figure 1). These short regulating RNAs have important biological functions, are limited in number and generally exhibit good stability and detection in clinical samples such as serum, plasma, urine and other biofluids. Reverse-transcription quantitative PCR (RT-qPCR) is the gold standard method for sensitive and specific quantification of microRNAs in cell-free biofluids. However, microRNA profiling in cell-free biofluids by RT-qPCR is also challenging in many ways. This is because cell-free biofluids

- contain very low amounts of RNA,
- potentially contain high levels of enzyme inhibitors,
- have an RNA composition that is highly susceptible to pre-analytical variations and low sample quality.

As a consequence, the quality of (even published) results on circulating microRNA data varies strongly, and many studies miss to use or provide sufficient information on required quality controls. In order to ensure your success in using RT-qPCR for microRNA profiling in cell-free blood samples, this technical note addresses the key challenges (Figure 2), and provides tips and solutions for successful microRNA experiments in serum and plasma.

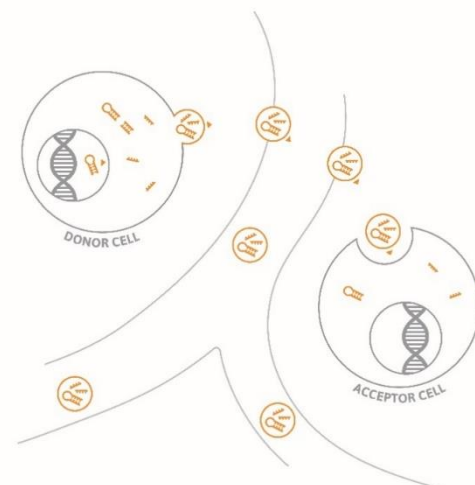
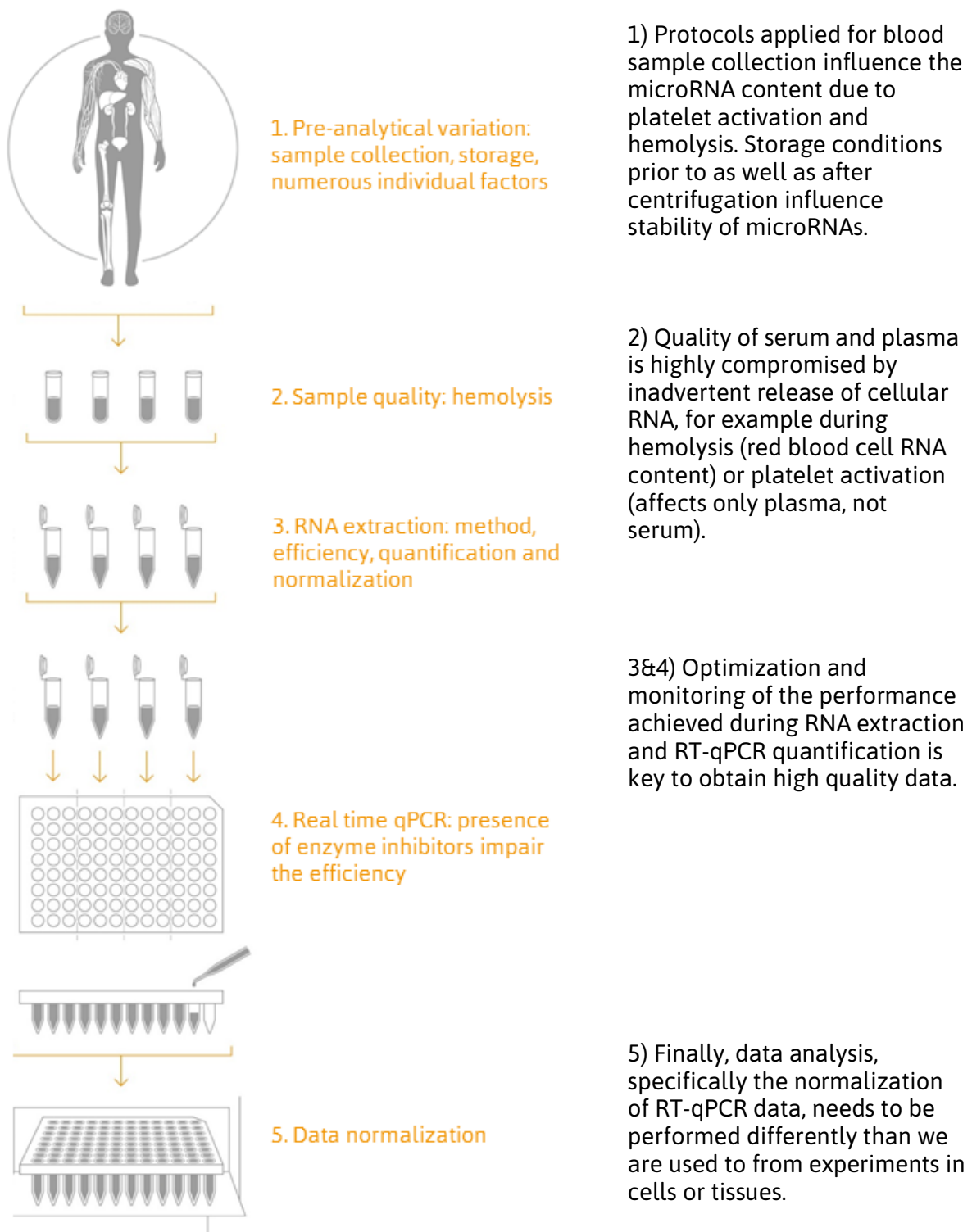


Figure 1: Circulating microRNAs are a novel class of bloodborne biomarkers. They are secreted from virtually any cell in the human body and distributed to other cells via the circulation. Local pathophysiologic processes in tissues can be detected using circulating microRNAs, and used for diagnosis and treatment monitoring of age-associated diseases.

Figure 2: Overview of the different steps of a microRNA experiment and potential key challenges:



1. Common sources of variability during microRNA analysis in cell-free biofluids

Pre-analytical variables are a common source for erroneous or misleading laboratory test results ¹. The detection of circulating microRNAs can be influenced by several factors, including the collection method along with specimen processing, storage conditions, and the composition of the specimen.

Table 1: Types, sources and mitigation of variability in qPCR experiments

Type of variability	Most important source	Mitigation
Pre-analytical variability	<p>Sample quality:</p> <ul style="list-style-type: none"> • Hemolysis affects both serum and plasma microRNA levels. • Platelet contamination or residual activation during sample collection can bias analysis of plasma samples. 	<p>Have a defined sample collection protocol (SOP) in place, which defines</p> <ul style="list-style-type: none"> - the choice of serum vs plasma and the rationale behind this choice, - the type of collection tube and anti-coagulant (plasma), - processing conditions such as centrifugation speed and time, temperature, incubation periods. <p>Ideally, test the sensitivity of biomarker candidates against platelet activation and hemolysis, by quantifying their concentration in platelets and red blood cells. Monitor hemolysis in all samples using endogenous controls or OD414 measurements.</p> <p>In case of heparin plasma samples, heparinase treatment of total RNA can be performed. However, ideally, other types of anticoagulants are used.</p>
Analytical variability	RNA extraction and reverse transcription.	RNA extraction and reverse transcription variability can be monitored using spike-in controls. This allows to normalize noise that was created during these steps, and to exclude samples with low data quality due to enzyme inhibition from the analysis. See Figure 3.
Biological variability	<p>Presence of enzyme inhibitors (for example heparin) in serum/plasma due the use of heparin as anti-coagulant or because of heparin treatment of patients.</p> <p>Anti-platelet therapy with aspirin®, clopidogrel, ticagrelor, etc. may alter microRNA profile.</p>	<p>Heparinase treatment of total RNA can be applied in case of known history of heparin treatment in subjects prior to blood collection (24 hours).</p> <p>Exclude patients currently undergoing anti-platelet therapy (if biomarkers are known to be present in platelets).</p>

Serum or plasma – does it make a difference?

Yes, it does make a difference. Processing of blood into serum means that platelets become activated during sample collection, before they and other blood cells are separated from the serum by centrifugation. During their activation, platelets release their intracellular microRNA content. Although the microRNA repertoire in platelets is not encompassing all human microRNAs, there are a number of highly abundant microRNAs also found in other cell types, such as miR-21-5p. Thus, microRNA analysis in serum is biased towards the platelet microRNA transcriptome ².

In contrast, processing of plasma means that whole blood is collected in the presence of an anticoagulant such as EDTA, citrate, or CTAD. Thereby, platelet activation is inhibited, resulting in a lower presence of platelet-derived microRNAs. The challenge with plasma collection is that incomplete removal of platelets during centrifugation or inadvertent/varying residual activation of platelets can add significant noise to the data. Therefore, plasma collection protocols need to be standardized, and ideally a two-step centrifugation protocol is used to minimize activation and contamination of platelets. Mussbacher et al. have shown clearly that EDTA compared to citrate or CTAD results in higher residual activation of platelets, which is likely to impact quantification of microRNAs as well ³.

Specimen collection: In a recent study, Kim et al. examined the influence of different blood collection tubes on the detection of microRNAs in plasma ⁴. They compared Vacutainer tubes (BD Diagnostics) containing EDTA, heparin, sodium citrate, sodium fluoride/potassium oxalate (NaF/KOx), or no anticoagulant and measured microRNAs by qPCR. Their data suggests that heparin tubes exhibit the lowest levels of microRNAs (which is due to the inhibitory activity of heparin on reverse transcriptases), with occasional improvement upon heparinase treatment.

→ Therefore, heparin should not be used as an anticoagulant.

Storage and stability: A crucial factor in the pre-analytical phase is the analyte stability, particularly in the context of an RNA species. Because of the critical influence of microRNA integrity for any downstream microRNA profiling technology, there is a strong need for good microRNA quality and high microRNA integrity ⁵. Mitchell et al. ⁶ demonstrated that plasma storage at room temperature for up to 24 h had minimal effect on selected microRNAs as measured by RT-qPCR. However, this observation was limited to a small set of microRNAs, and the effect of sample incubation prior to centrifugation (i.e. separation of cells from cell-free component) was not tested. In addition, no information on analytical variability was provided, therefore the minimum detectable effect sizes were not known.

→ We recommend to centrifuge whole blood within 2 hours of sample collection, and to store centrifuged serum or plasma immediately at -80°C.

Recently, it was shown that microRNA levels obtained from exosomes isolated from fresh plasma were significantly lower compared to exosome isolations from plasma that had been frozen and thawed ⁷. These findings indicate the importance of the freezing cycle and the time when experiments are performed.

Biological variance: Another very important variable, and undoubtedly the least standardizable, is the individual itself. Since microRNAs are deeply entwined in regulatory networks, their levels are prone to be influenced by both intra-individual and external factors, including diet, exercise, age and race. Understanding the degree of biological variation in microRNAs is imperative for epidemiological and clinical research, particularly with respect to investigating microRNAs for disease risk assessment. There are only few studies evaluating the intra-individual variability of circulating microRNAs over time ⁸⁻¹⁰. The bottom line is that when publishing results from microRNA studies, it is pivotal to provide a comprehensive description of the preanalytical and analytical factors used, in order to allow for others to perform the exact same experiments in validation studies. Heegaard et al. ¹¹ identified circulating microRNAs that undergo clear rhythmic fluctuations in abundance during a 24-hour period in a group of 24 healthy young male individuals. Moreover it was shown that microRNAs are uniquely and dynamically regulated in response to acute exhaustive exercise and sustained aerobic exercise training ¹².

We recommend to evaluate whether the following in-/exclusion criteria can be included when designing circulating microRNA experiments:

- Fasted blood draw between 8-10am in the morning to prevent diurnal variations.
- Any type of medical treatment - in elderly populations, specifically treatment with heparin in the last 24 hours - should be captured with a questionnaire.
- Renal function should not be impaired. We recommend an eGFR cut-off of 30 ml/min.
- Hormones such as estradiol have downstream effects on microRNA transcription. Thus, age and gender have an impact on circulating microRNA levels. This needs to be considered when planning case/control studies.

2. Sample Quality: Hemolysis

Hemolysis can be a major cause of variation in serum/plasma microRNA levels due to contamination with cellular RNA ¹³. Three studies identified miR-16 and miR-451a as being the most highly abundant microRNAs in red blood cells (RBCs), and found levels of these microRNAs in plasma to be most affected by hemolysis ¹³⁻¹⁵. As part of their study on blood cell counts, Pritchard et al. also examined the effect of hemolysis on 10 microRNAs, and found that RBC-associated microRNAs were increased by 20 to 30-fold in hemolyzed plasma. One of these 10 microRNAs was miR-92a, a proposed colon cancer biomarker ¹⁶. Hemolysis is relevant in cancer patients because they have an

increased predisposition for hemolytic disorders. McDonald et al. spiked non-hemolyzed serum samples with hemolysates from the corresponding RBCs ¹⁵. When they correlated hemoglobin concentrations, they found that highly RBC-expressed miR-15b, miR-16 and miR-24, were increased in a dose-dependent fashion. At the same time miR-122, a predominantly liver-specific microRNA, was not affected by hemolysis ¹⁷.

The presence of hemolysis should be assessed visually for each sample. In addition, hemolysis can be determined using the hemolysis index, which relates the level of a microRNA highly expressed in red blood cells (miR-451a), with a microRNA unaffected by hemolysis (miR-23a-3p) ¹⁸. Blondal *et al.* found that delta Cq (miR-23a-3p - miR-451a) is a good measure of the degree of hemolysis, where values of more than five is indicative of possible erythrocyte microRNA contamination, and a delta Cq of > 7 or more points at a high risk of hemolysis affecting the data obtained in human samples. Note that the values are different in mouse and rat biofluid samples, and might differ based on the RT-qPCR platform used.

Another convenient option to determine hemolysis is the measurement of the absorbance peak of free hemoglobin by assessing free hemoglobin using a spectrophotometer such as NanoDrop™. Human serum or plasma samples are classified as being hemolyzed if the absorption at 414nm is exceeding 0.2. However, the presence of small amounts of cellular contamination in serum or plasma samples is not readily detectable by visual or spectrophotometric means.

Tips on how to avoid hemolysis ¹⁹:

- Use good and consistent sample collection devices throughout a study (e.g. BD Vacutainer),
- Follow manufacturer's instructions,
- Avoid drawing blood from a hematoma,
- Avoid foaming of the sample,
- Make sure the venipuncture site is dry,
- Avoid a probing, traumatic venipuncture,
- Avoid prolonged tourniquet application or fist clenching,
- Use correct size needle (~22 gauge),
- Fill vacuum tubes completely.

3. The RT-qPCR workflow: RNA extraction, normalization and quality control

There are numerous challenges when it comes to isolating and purifying circulating or exosomal RNA from bodily fluids such as plasma, serum and urine:

- (1) There is usually a very small amount of RNA present, and hence larger input volumes (> 1 ml) are advantageous. Co-purification of enzyme inhibitors, limited sample availability (especially in case of retrospective studies), and challenging scale-up of extraction methods such as phenol-chloroform extraction or purification using micro centrifuge-based spin column chromatography are the consequences.
- (2) RNA isolated from bodily fluid contains inhibitors for many downstream applications ²⁰. For example, heparin, which is used to collect non-coagulating blood for plasma processing, and high levels of hemoglobin, lactoferrin, and IgG in blood, or urea in urine, are known to inhibit PCR enzymes.
- (3) Current methods for exosome isolation by precipitation, ultrafiltration or ultracentrifugation are lengthy.

RNA extraction kit: method of choice

The principles for isolation of microRNAs are generally the same as for isolation of total RNA, except that microRNA isolation protocols are slightly modified to retain (and sometimes enrich) the small RNA fraction. Widely used commercially-available products such as miRNeasy (Qiagen), *mirVana*[™] (Ambion), and PureLink[™] (Invitrogen) microRNA isolation kits are based on chemical extraction using concentrated chaotropic salts such as guanidine thiocyanate (e.g. Trizol and QIAzol[®] reagents) followed by a solid-phase extraction procedure on silica columns ¹⁴. However, different extraction kits have varying efficiency at extracting different microRNAs, often resulting in variances in microRNA expression profiles ^{14,21}. Therefore, it is recommended to utilize a single extraction kit and buffer throughout a study where direct comparisons of microRNA levels are intended.

The TAmiRNA Serum/Plasma RNA extraction kit, which is part of all TAmiRNA one-stop kit solutions such as osteomiR[™], thrombomiR[™] and toxomiR[™], was designed to enable optimal recovery of total RNA from cell-free biofluids.

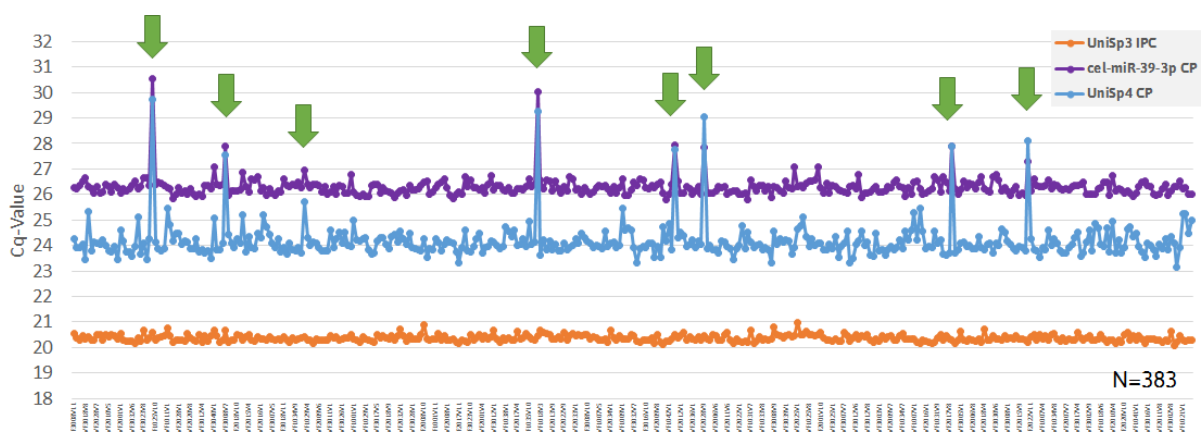
Synthetic spike-in controls are essential for good quality control

Spike-in controls give valuable insight into the quality of a qPCR experiment, and should be used to monitor the efficiency and variability introduced during the main steps of a RT-qPCR experiment. They can be used to identify outliers due to the

presence of inhibiting factors (e.g. heparin, urea or bilirubin) or incorrect handling (Figure 3). Uniform Cq-values obtained for spike-in controls demonstrate successful and homogenous RNA isolation, reverse transcription and PCR across all samples. However, synthetic spike-ins do not reveal the RNA content and quality in the biological sample. The TAMIRNA RT-qPCR workflow relies on the following spike-in controls:

- **RNA Spike-ins:** Synthetic RNA spike-ins are added to the lysis buffer during RNA extraction. These controls give information about the overall variability in the RT-qPCR workflow and can detect samples where RNA extraction has failed.
- **RT Spike-ins:** Synthetic RT spike-Ins such as non-mammalian “cel-miR-39” are added to the reverse transcription mastermix. It gives information about the variance arising during reverse transcription and PCR amplification, and detects samples where RT failed or was inefficient due to presence of enzyme inhibitors. It shares the natural microRNA sequence from *C. elegans*, which is not found in mammalian species. Reverse transcription efficiency is known to introduce the highest technical variance to RT-qPCR data.
- **PCR Spike-ins:** Synthetic DNA oligos are incorporated at a fixed position on every TAMIRNA test qPCR plate. They are used to monitor PCR efficiency and to detect the presence of PCR inhibitors.

Figure 3: Spike-in controls for monitoring the variability during RNA extraction, reverse transcription and PCR amplification. Shown are the raw Cq-values (Roche LC480 II, second-derivative maximum) obtained in a study with 383 serum samples. Three types of spike-ins are shown: orange = PCR spike-in for PCR variability, CV < 10%; purple = reverse-transcription (RT) spike-in for RT and PCR variability, CV < 15%; blue = RNA spike-In for RNA extraction, RT, and PCR variability, CV < 25%



The results obtained for all three spike-ins need to be evaluated carefully. They can be very useful to identify potential outliers, and to exclude samples with low data quality from statistical analysis. Spike-ins can further be used for normalization of Cq data of

informative microRNAs to remove technical variance. A value outside the acceptable range indicates that the analysis has failed, and that the sample is not valid. The acceptable range is based on assay validation runs using different reagent lots and operators.

Data normalization for microRNA analysis in cell-free biofluids

The goal of every normalization is to minimize variation that can mask or exaggerate biologically meaningful changes, thereby increasing the accuracy of expression measurements. The choice of a normalization strategy, however, is anything but trivial. The purpose of many microRNA RT-qPCR expression experiments is to identify differences between two or more groups of samples, such as a control and a diseased specimen. Thus, the intention of normalization is to remove as much variation as possible between groups except for that difference that is a consequence of the disease state itself.

When working with cells or tissues, the use of so-called “reference” or “housekeeping” genes is common practice. The assumption is that these genes are expressed at very constant rate and independent of changes in the physiologic state. Hence, they allow us to account for differences in total RNA input.

When we use cell-free biofluids for RNA analysis, accurate quantification of extracted RNA concentrations is not always possible and reliable, due to the very low concentration and potential contaminations. However, compared to experiments with cell or tissues, the input amounts - i.e. the volume of biofluid - can be standardized. We regularly use precisely 200 µl serum or plasma per sample. Under the assumption that the standardized procedures of RNA extraction, reverse transcription and PCR amplification have equal efficiencies across all samples, no normalization would be required before analysis. We monitor this assumption by adding spike-in controls at every step of the workflow (see above). This enables us to capture the workflow variability and eventually perform normalization to reduce analytical variability. For this purpose, we use the Cq values obtained from RNA spike-in controls, because they reflect the overall variability introduced by RNA extraction, reverse transcription and PCR amplification. In our experience, most variability is introduced during reverse transcription.

4. Reverse-transcription quantitative PCR analysis (RT-qPCR)

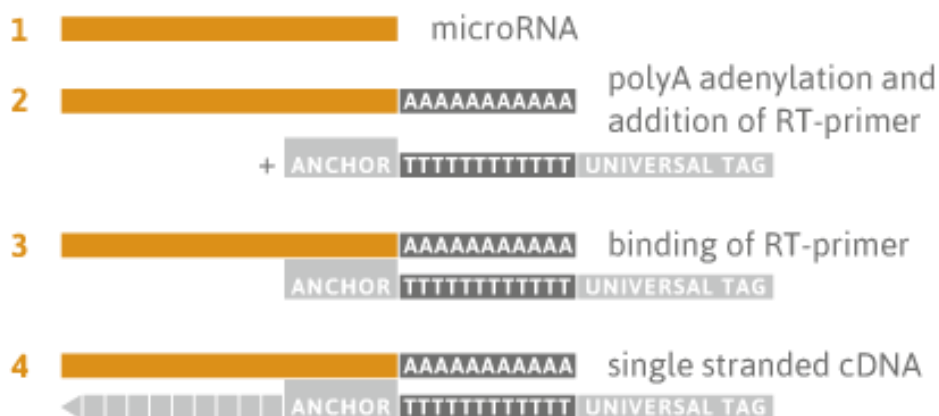
The challenge with PCR analysis of microRNAs is their short size and variable GC content, which complicates the harmonization of primer annealing temperatures for different microRNA sequences in one PCR run. Chemical modifications of primers or probes such as locked nucleic acids (LNA™) have been shown to increase specificity significantly compared to other chemistries²². LNA-enhanced primers can effectively distinguish microRNA sequences with just a single nucleotide difference and maintain high PCR efficiency, resulting in optimal sensitivity.

Another challenge is the analytical variability of reverse transcription (RT) reactions due to enzyme performance or sample matrix effects. Therefore “universal RT reactions” have an advantage over target specific RT reaction in terms of robustness and reproducibility, and allow for parallel analysis of hundreds of microRNAs from the same cDNA sample. Using TAmiRNA RT-qPCR chemistry, universal RT is achieved in a protocol consisting of polyA adenylation, followed by RT using an anchored oligo (dT) reverse primer (Figure 4, step 1). During qPCR, the combination of LNA-enhanced template-specific forward and reverse primers ensures sufficient sensitivity (no pre-amplification) and specificity (Figure 4, step 2).

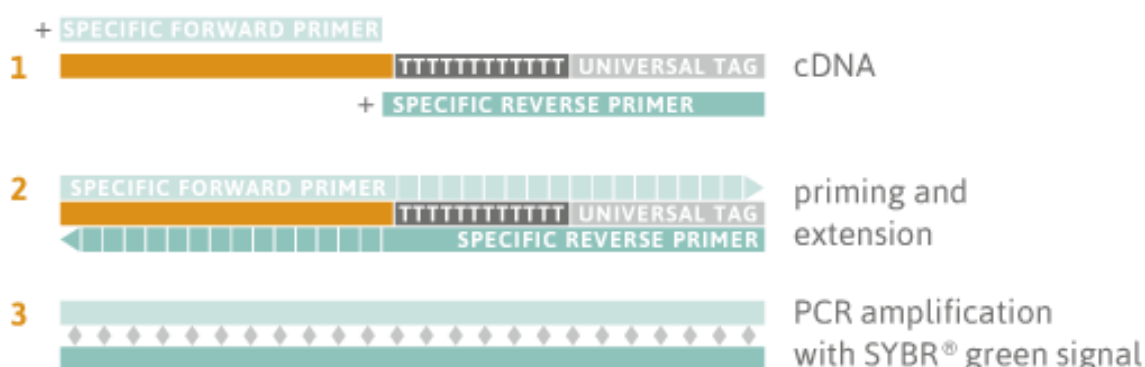
Using this protocol, TAmiRNA’s all-in-one kits enable rapid parallel analysis of microRNA signatures such as osteomiR™ and thrombomiR™ with high sensitivity and specificity. In addition, primer-coated PCR plates accelerate preparation of PCR mastermixes, , and reduce variability during screening of hundreds of microRNAs and validation of selected candidates.

Figure 4: RT-qPCR workflow using TAmiRNA’s osteomiR™ or thrombomiR™ chemistries. Starting from extracted total RNA including microRNAs, universal reverse transcription is achieved by polyA adenylation followed by reverse transcription using an anchored oligo(dT) primer with a universal tag. In the second step, microRNA-specific qPCR amplification is performed using specific forward and reverse primers, which is enabled by incorporation of locked-nucleic acid modifications (LNA) at specific sites.

Step 1: Reverse transcription (RT)



Step 2: Real-time PCR amplification



5. Data analysis of exploratory studies: important steps from differential expression to development of diagnostic tests.

Data analysis begins by choosing a suitable experimental design. We strongly recommend to engage with biostatisticians as early as possible to define experimental groups and sample sizes, and to draft a statistical analysis plan.

The first step of every data analysis is quality control. For that purpose, we recommend to plot the C_q-values of spike-in controls (see Figure 3) to identify samples with risk of low data quality, and to determine overall variability in the data set. In addition, the presence of hemolysis needs to be evaluated using appropriate measurements (see section 2 on sample quality).

In case of missing values, i.e. qPCR signals below the limit of detection, it needs to be decided whether to keep or discard such low abundant microRNAs from the data set. Large enough data sets allow for imputation strategies, which estimate missing values from the available data, thus enabling statistical analysis. However, the impact of imputation should be carefully evaluated. Keep in mind that low abundant microRNAs will always exhibit higher technical variability compared to high abundant microRNAs, and will potentially complicate subsequent validation studies and assay development.

After QC and normalization, we recommend to perform an exploratory analysis such as principal component analysis (PCA) or clustering that is visualized as a heatmap. These analyses can be performed using either the entire set of microRNAs or subsets that show the highest variability. PCA and clustering are helpful to estimate the effect of experimental factors on circulating microRNA levels, and to build hypotheses that can be followed in subsequent statistical analysis.

Differential expression analysis between experimental groups is commonly performed to identify microRNA candidates with effect sizes (fold changes) that are not random but biologically relevant. We recommend to use p-value adjustments for multiple testing in order to avoid a large number of false positives in exploratory studies. At this stage, we usually want to test whether observed effect sizes are balanced (same number of up- and down-regulated microRNAs) or biased in one direction. Interestingly, we frequently observe such systemic effects for circulating microRNA experiments. By applying cut-offs for fold change and p-value, promising microRNA biomarker candidates are identified and can be followed up in validation experiments as well as for functional characterization.

At this stage, one of the main difficulties in translating results from laboratory into clinical practice seems to be in the multivariate analysis, which ideally leads to an optimal (robust) combination of microRNAs into a “classifier”. The advantage of multivariate modeling is that it uses more biological information to provide more sensitive and specific information about disease phenotypes, while at the same time providing a readout (e.g. score) that is useful for decision makers. Thus, from this perspective the question is ‘how to combine microRNAs appropriately?’

At TAmiRNA, we have several advanced IVD (In-vitro diagnostics) development projects where multivariate analyses have been performed.

osteomiR™: We have collaborated with Swiss company SimplicityBio (<http://www.simplicitybio.com>) for the development of the osteomiR™ algorithm. The model employs the principals of Fuzzy Logic and is rule-based. This means that the algorithm is a “white box”, which means that it can be explained and followed (in comparison to a black box). The algorithm uses information from 10 microRNAs, converts them into microRNA pairs and feeds the resulting values into the rule-based algorithm. The algorithm returns a single-indexed score between 0 and 1, which continuously describes fracture risk in a subject. By defining cut-offs for this score, we can stratify patients into high, medium or low risk groups.

hepatomiR™: In this case, we have used a logistic regression model to compute a p-score (probability score) based on the information of three microRNAs that are converted into two microRNA pairs. Using the discovery data set, two cut-offs with different stringency were established and subsequently validated in independent data sets. We have recently published the discovery and validation of microRNA biomarkers for prognosis of liver failure using this approach ²³.

Generally, the lower the complexity of a model, the better. Highly complex models such as decision trees may result in overfitting to the training data, which impacts robustness and performance in validation sets.

Therefore, we recommend to compare different modelling approaches (i.e. LogR, SVM, decision-trees, LASSO, etc.) and choose the most robust method that gives acceptable performance. Many of these tools also allow to penalize the size of the model because the bigger a model, the higher the risk of overfitting.

6. Conclusion

The identification of stable circulating microRNAs in various disease states offers the potential for discovery of novel biomarkers and new biological insights. As outlined herein, important considerations regarding the low abundance of microRNA in plasma/serum and potential contaminants associated with these sample types must be addressed to ensure reliable results.

Using the guidelines outlined in this tech note, reproducible and relevant data can be obtained.

We at TAmiRNA are specialized in technologies for profiling levels of blood-circulating microRNAs and developing multi-parametric classification algorithms (“signatures”). TAmiRNA uses these technologies to develop minimal-invasive diagnostic tests for drug development, early diagnosis and prognosis of disease, and as companion diagnostic tests to support treatment decisions.

If you have further questions, do not hesitate to [contact us](#). We are happy to help you successfully complete your microRNA project.

7. References

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