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Review Article

Assessing sample and miRNA profile quality in serum and plasma or other biofluids

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ABSTRACT

MicroRNAs (miRNAs) constitute a class of small cellular RNAs (typically 21–23 nt) that function as post-transcriptional regulators of gene expression. Current estimates indicate that more than one third of the cellular transcriptome is regulated by miRNAs, although they are relatively few in number (less than 2000 human miRNAs).

The high relative stability of miRNA in common clinical tissues and biofluids (e.g. plasma, serum, urine, saliva, etc.) and the ability of miRNA expression profiles to accurately classify discrete tissue types and disease states have positioned miRNA quantification as a promising new tool for a wide range of diagnostic applications. Furthermore miRNAs have been shown to be rapidly released from tissues into the circulation with the development of pathology.

To facilitate discovery and clinical development of miRNA-based biomarkers, we developed a genome-wide Locked Nucleic Acid (LNA™)-based miRNA qPCR platform with unparalleled sensitivity and robustness. The platform allows high-throughput profiling of miRNAs from important clinical sources without the need for pre-amplification.

Using this system, we have profiled thousands of biofluid samples including blood derived plasma and serum. An extensive quality control (QC) system has been implemented in order to secure technical excellence and reveal any unwanted bias coming from pre-analytical or analytical variables. We present our approaches to sample and RNA QC as well as data QC and normalization. Specifically we have developed normal reference ranges for circulating miRNAs in serum and plasma as well as a hemolysis indicator based on microRNA expression.

1. Introduction

microRNAs (miRNAs) constitute a class of small RNAs that function as post-transcriptional regulators of gene expression [1]. Although they are relatively few in number (<2000 human miRNAs), current estimates indicate that more than one third of the cellular transcriptome is regulated by miRNAs [2]. Unsurprisingly therefore, miRNAs play important regulatory roles in most cellular and developmental processes and have been implicated in a large number of human diseases [3,4]. Since the discovery of extracellular and circulating microRNAs a few years ago, the study of microRNAs in biofluids such as serum, plasma, urine and cerebrospinal fluid has rapidly expanded [5]. Due to their wide-ranging biological potential and the fact that miRNAs seem to be relatively stable in readily available biofluids, these small 21–23 nt molecules are prime candidates for use as non-invasive biomarkers in molecular diagnostics of disease and other clinical conditions such as organ damage as well as pre-clinical toxicology and drug safety assessments [6]. Indeed numerous studies have shown that secreted microRNAs can be implicated in pathogenic conditions such as various cancers, coronary heart disease and organ damage [7–10].

The precise role of circulating miRNAs is still largely unknown. Circulating or extracellular miRNAs have been shown to be stabilized and protected from RNAse degradation by inclusion in various protein complexes or membranous particles such as exosomes or microvesicles [11–14]. There does seem to be a subset of cell-free microRNAs present in normal blood with possible functions within the circulatory and immune systems [15]. The miRNA profile in serum and plasma has been shown to reflect disease states such as cancer [16] as well as organ damage and injury [17]. It has also been shown that miRNA containing subcellular vesicles can be taken up by cells and cause changes in cellular gene expression during pathological conditions [18,19]. These findings suggest a biological function for extra-cellular miRNA yet to be fully described.

Abbreviations: LNA, (Locked Nucleic Acid); qPCR, (quantitative real time PCR); RT, (reverse transcription); QC, (quality control); miRNA, (microRNA); Cq, (quantification cycle).

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From the analytical viewpoint miRNAs in serum and plasma have been shown to be stable for up to 48 h even when stored at room temperature and are also stable after multiple freeze–thaw cycles (in house data). However the accurate and robust measurement of miRNAs in biofluids is made challenging by a number of factors. Firstly the miRNAs themselves are very short and consist of highly divergent sequences with large variation in GC content. This variability leads to very different hybridization properties between different miRNA sequences and makes simultaneous measurement of all miRNAs challenging. Although miRNAs are highly divergent in general, individual members of miRNA families are highly similar, sometimes varying only by a single nucleotide. Secondly, the overall amount of miRNA, and RNA in general, that is present in samples such as serum and plasma is very low. Reproducible and robust isolation of miRNAs from serum and plasma can be achieved by the addition of carrier RNA during the isolation procedure [20]. The use of a highly sensitive, specific and accurate miRNA qPCR method that allows linear detection of miRNAs even at very low target concentrations is also essential. Both of these challenges can be overcome by the use of short LNA™-enhanced, microRNA specific primers for detection of reverse transcribed miRNAs [21].

To move microRNA discovery from the molecular biology lab to the clinic requires not only a highly reliable and reproducible assay system, but also requires that pre-analytical and analytical variables are easily identified and accounted for by thorough quality control of the samples being analyzed. Sampling, and sample preparation as well as RNA isolation and storage can be sources of pre-analytical variation. A number of recent papers have discussed various pre-analytical variables for miRNA profiling in serum and/or plasma [22–24]. These studies have shown that cellular contamination and hemolysis of plasma (and serum) samples can be a major cause of variation in miRNA levels not related to any biological difference. A common source of analytical variation is the co-purification of qPCR inhibitors known to be present in serum and plasma in the RNA sample itself.

We have developed a highly sensitive and accurate miRNA qPCR system which has been shown to be especially well suited to the analysis of biofluid samples with limited RNA content, such as serum and plasma [25]. The miRNA assays are all (95%) validated to be linear down to 10 copies of microRNA target in the PCR reaction (equivalent to 4000 copies in a 20 μl cDNA synthesis reaction) which is essential for achieving accurate miRNA measurements without the potential biases which can be introduced by pre-amplification.

Using this system we have profiled miRNA from over 1500 serum and plasma samples. The samples used have come from a large number of different sources and have therefore been subjected to extensive quality control measures both before RNA isolation and on the RNA itself in order to remove any bias which could be linked to e.g. sampling methods, storage or purification. Successful biomarker discovery projects are dependent on controlling for these sources of pre-analytical variation. As a result we have developed a normal reference range for 119 miRNAs in serum and plasma as well as an RNA QC panel consisting of assays for both endogenous miRNAs and spiked-in RNAs for a complete QC of RNA samples. In addition we have developed a hemolysis indicator based on two endogenous microRNAs for use with RNA samples or archival data.

2. Material and methods

2.1. RNA isolation

Total RNA was extracted from serum using a commercial column-based system following the manufacturer's instructions with the following modifications (Qiagen miRNeasy® Mini Kit). Serum or plasma was thawed on ice and centrifuged at 3000×*g* for 5 min in a 4 °C microcentrifuge. An aliquot of 200 μl of serum/plasma per sample was transferred to a new microcentrifuge tube and 750 μl of a Qiazol mixture containing 1.25 μg/mL of MS2 bacteriophage RNA (Roche Applied Science) and spike-ins were added to the sample. A rinse step (500 μL Qiagen RPE buffer) was repeated 2X. Total RNA was eluted by adding 50 μL of RNase-free water to the membrane of the spin column and incubating for 1 min before centrifugation at 15,000×*g* for 1 min at room temperature. The RNA was stored at −80 °C.

2.2. RNA spike-in

The RNA spike-in kit (Exiqon A/S) was used according to the manufacturer's instructions.

2.3. cDNA synthesis and real time PCR

8 μl of RNA eluate was reverse transcribed in 40 μl reactions using the miRCURY LNA™ Universal RT cDNA Synthesis Kit (Exiqon). The cDNA was diluted 50 x and assayed in 10 ul PCR reactions according to the protocol for the miRCURY LNA™ Universal RT microRNA PCR System (Exiqon A/S); each microRNA was assayed once by qPCR on the Serum/plasma Focus microRNA PCR panel. A no-template control (NTC) of water was purified with the samples and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cp (by the second derivative method) and for melting curve analysis.

2.4. Data filtering and analysis

The amplification efficiency was calculated using algorithms similar to the LinReg software [26,27]. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for each particular assay. Furthermore any sample assay data point must be detected with 5 Cps less than the corresponding negative control assay data point, and with a Cp < 37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis.

3. Results and discussion

3.1. Establishing a normal reference range of miRNA expression in serum and plasma

Recent reports have suggested that a common source of unwanted bias in miRNA profiles in serum and plasma samples is cellular contamination of the samples with various blood cell types [22]. It is vital for any biomarker discovery project utilizing cell free microRNA from serum or plasma that the cellular content of the biofluid is minimized, that miRNA expression levels are interpreted in light of blood cell counts, or that miRNA biomarkers are sought among miRNAs that are not expressed in blood cells. Minimizing cellular content can be done by standardizing the sampling procedure as well as the handling of the material. The National Cancer Institute Early Detection Research Network (EDRN) has recently published standard operating protocols for the sampling procedure including serum and plasma [28]. After sampling it is important to treat the biofluids according to instructions of the sampling tube manufacturer. Separation of the red blood cells, buffy coat and the plasma must be done carefully to not disturb the buffy coat. After collection of plasma we recommend spinning once again to minimize buffy coat contamination.

For serum this step is not necessary due to the coagulation of the sample.

The presence of small amounts of cellular contamination in serum or plasma samples is not readily detectable by visual or spectrophotometric means. The effect on the miRNA expression profile in the resulting RNA samples depends on the amount of cellular carry over and the precise cell types and will therefore be wide ranging. In order to identify samples with potential cellular contamination, we identified the 119 microRNAs that are most commonly present in serum and plasma samples (see material and methods for details of data QC and filtering, see appendix for the list of microRNAs). The results of microRNA profiling on 381 samples that passed standard quality control were used to generate a mean qualification cycle value for each of the 119 microRNAs. Fig. 1A shows the mean Cq values for this normal reference range of miRNA expression in serum/plasma using the miRCURY LNA™ microRNA PCR system as well as a representative sample which was known to be of high quality and free from cellular contamination. It is clear that although the representative plasma sample varies from the mean values, the overall miRNA expression levels are similar.

In order to be able to identify outlier samples as well as to control for cellular contamination, we generally compare all miRNA profiles obtained from serum and plasma with the reference range values. Fig. 1A also shows an example of a compromised plasma sample that is probably contaminated with blood cells. The overall miRNA levels in the sample are clearly different from the mean values. The comparison in Fig. 1A is done before normalization, but normalizing the miRNA profiling data using a geometric mean of all values does not rescue the compromised sample from being recognized as being significantly different from the reference sample (Fig. 1B–C). When comparing miRNA profiles from serum or plasma samples it is important that the samples are as technically similar as possible in order to optimally detect the biological variation of interest. Samples with very different miRNA expression profiles from the rest of the samples in the project should not be included in the final data analysis.

3.2. Controlling hemolysis in RNA samples and archival data

Another recently reported source of significant variation in miRNA profiles from serum/plasma is hemolysis (rupturing of erythrocytes) [21]. Hemolysis can occur in most steps of the sampling and handling procedures until serum/plasma has been separated from the rest of the circulatory components. Monitoring hemolysis in serum and plasma samples can however be done by various methods that measure free hemoglobin levels. A simple and cost effective way is to use a spectrophotometer and measure oxyhemoglobin absorbance at $\lambda = 414$ nm. Distinct absorbance peaks can be used to disqualify hemolysis samples before time consuming and costly downstream sample processing takes place. An example of spectrophotometric analysis of representative serum and plasma samples affected by hemolysis is shown in Fig. 2A.

However, if the original serum or plasma sample is no longer available and only purified RNA is available, an alternative method to identify putative hemolyzed samples is to use miRNAs known to be enriched in erythrocytes like miR-451 or miR-144 [29]. As the level of these miRNAs can also be influenced by other factors such as pre-clinical variables, disease state, tissue/organ damage and natural variation, a simple threshold cannot be used as an indicator of hemolysis. The level of the erythrocyte specific miRNA must be related to a miRNA found to be un-affected by hemolysis and this will allow identification and, if required, elimination of outlier samples.

In our experience miR-23a is relatively stable in plasma and serum and is not affected by hemolysis. We have found that a delta Cq (miR-23a-miR-451) of more than five is an indicator of possible erythrocyte miRNA contamination, and a delta Cq of 7–8 or more indicates a high risk of hemolysis affecting the data obtained (Fig. 2B). In our quality control process for serum and plasma samples, we use this hemolysis indicator to (1) monitor sampling protocols and sampling sites (2) disqualify outliers (e.g. samples with highly different delta Cq (miR-23a-miR-451) values) and (3) monitor that hemolysis is not affecting specific sample groups being interrogated. This hemolysis indicator can also easily be applied to archival data.

It is important to note that many miRNAs in serum and plasma are not affected by hemolysis and therefore it may still be possible to detect disease associated miRNA biomarkers even from samples affected by hemolysis. However it is crucial to be aware of the possible effects on the miRNA profile when performing normalization and data analysis so that any systematic bias can be eliminated. We have shown that applying a pre-set cut-off for indications of hemolysis on a dataset from clinical plasma samples could improve the sensitivity and specificity of a miRNA signature for early colorectal cancer significantly ([30] and unpublished data).

3.3. A qPCR based RNA QC method

A third source of possible variation in miRNA profiles from serum and plasma is the RNA isolation procedure and specifically the presence of inhibitors that affect the cDNA synthesis and/or real-time PCR reactions. As cell-free miRNA concentrations are low in these samples, it is tempting to try to maximize the amount of sample used per reaction. However, increasing the amount of RNA input could also lead to an increased concentration of inhibitors which may be derived from the biofluid or introduced via reagent carry-over during sample preparation and suboptimal RNA isolations. Variation in RNA isolation efficiency is difficult to identify as the yield of RNA from serum and plasma is very low and robust RNA purification therefore requires the addition of carrier. This means that the amount of miRNA in the samples cannot be accurately measured and used to determine input amounts.

In order to control for sample specific effects on assay performance we have developed a set of synthetic oligonucleotides that share common sequence features with miRNAs (short length and variable GC content from 43–50%). A subset of these synthetic RNA templates can be spiked into the serum or plasma samples during the RNA isolation procedure via the lysis buffer in three different concentrations. This subset of spike-ins is used to measure and compare the yield from different RNA extractions. A second RNA spike-in is used to monitor the efficiency of the cDNA synthesis reaction for signs of inhibitors and a third DNA spike-in is used to monitor the real-time PCR reaction. A no-template control (NTC), that is a water sample that has been taken through the RNA isolation process like the other samples including the addition of spike-ins, is also recommended. The NTC is then profiled to monitor the baseline for the spike-ins and can be used to reveal potential inhibitions. Fig. 3 shows an example of results from the set of RNA and DNA spike-ins on two plasma samples.

The use of a set of spike-in templates during RNA isolation combined with a carefully selected set of assays targeting endogenous miRNAs can be used as a qPCR-based quality control procedure for RNA samples from biofluids including serum and plasma. The spike-in templates are used to monitor yield and possible contamination by inhibitors. Expression patterns of endogenous miRNAs can be used to identify putative outliers and samples affected by hemolysis or inhibition. An up-front quality control of RNA from serum and plasma using a small panel of assays can thereby be used to monitor important sources of pre-analytical and analytical variables and ensure high quality data from the subsequent miRNA profiling study.

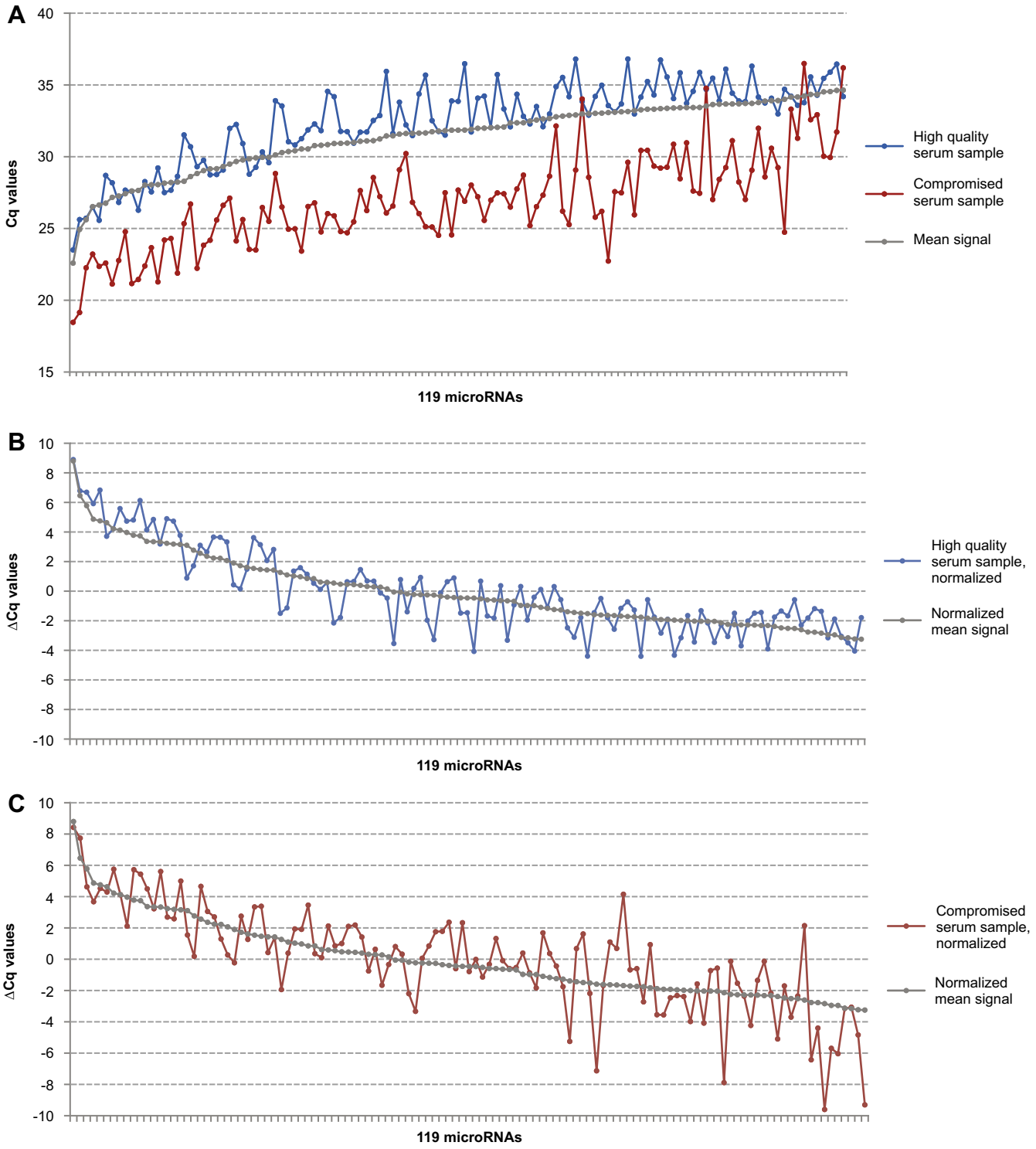
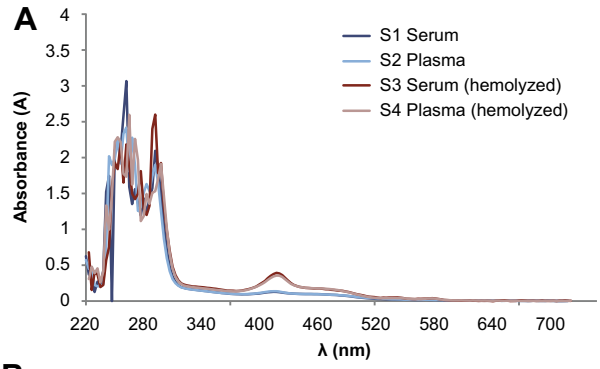


Fig. 1. Using a reference range of miRNA expression to identify suboptimal serum and plasma derived RNA. (A) Mean quantification cycle (Cq) values for 119 human miRNAs (sorted in ascending order) based on 381 high quality serum and plasma samples (dark gray). Raw Cq values from a representative high quality plasma sample is shown in blue and raw Cq values from a compromised plasma sample is shown in red. The ΔCq values from the mean signal and the high quality sample as well as the compromised sample are shown after normalization using the geometric mean of all 119 miRNAs (B and C respectively).

4. Conclusions

miRNAs present in biofluids such as plasma and serum show great promise as minimally invasive biomarkers for diverse pathological conditions. Although they have great promise, miRNAs in biofluids are very challenging to measure accurately and pre-analytical and technical factors can easily affect the miRNA levels resulting in biases that do not reflect the biological state of the samples. We have described a versatile LNA™-based qPCR method for genome-wide profiling of miRNAs with absolute sensitivity,



B

Sample	Cq values		dCq (miR-23a - miR-451)
	miR-23a	miR-451	
S1 Serum	29,05	24,15	4,90
S2 Plasma	28,65	24,53	4,13
S3 Serum	30,05	21,55	8,50
S4 Plasma	29,56	21,93	7,64

Fig. 2. A miRNA-based hemolysis indicator. (A) Absorbance spectrum of representative non-hemolyzed and hemolyzed serum and plasma samples obtained using NanoDrop measurements. Hemolyzed samples show a peak at 414 nm. (B) Table of Cq values for miR-23a and miR-451 from the same samples, hemolyzed samples have a higher miR-23a-miR-451 delta Cq (dCq).

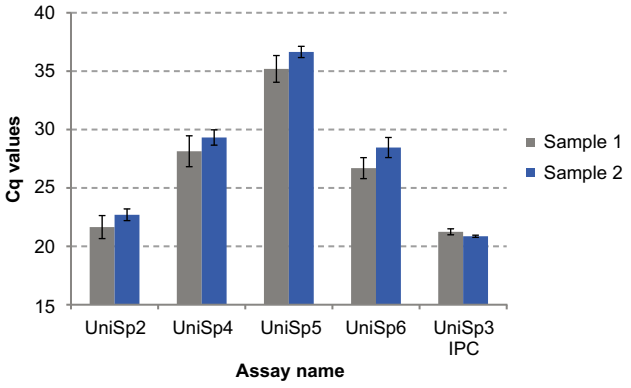


Fig. 3. The use of synthetic spike-ins to control RNA isolation quality. Three synthetic RNA spike-ins in different concentrations are used to control yield (UniSp 2, UniSp 4 and UniSp5). A fourth Spike-in, UniSp6 is used to monitor the cDNA synthesis reaction for signs of inhibition and UniSp3 IPC is used to monitor PCR efficiency. Each plasma sample has been extracted five times and error bars show the standard deviation between extractions. A significant difference in the Cq values for UniSp6 indicated the possible presence of RT inhibitors in sample two.

accuracy and ease-of-use that is optimal for measuring miRNAs in biofluids and other clinical samples.

We have strengthened this PCR system with an extensive QC and data analysis pipeline that has been developed and implemented in order to facilitate biomarker discovery and assay development studies by identifying and eliminating pre-analytical and technical factors in samples. By monitoring the Cq profile of the 119 miRNAs most commonly expressed in serum and plasma, we can quickly identify samples affected by sources of pre-analytical variation such as cellular contamination. The relative expression of the erythrocyte specific miR-451 and the stable miR-23a can be used as an indicator of hemolysis in RNA samples or miRNA profiling data from serum and plasma samples. Finally, a minimal set of qPCR assays for synthetic and endogenous targets can be used as a qPCR based method for quality control of RNA samples isolated from biofluids.

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Appendix A

Table of 119 miRNAs most commonly found in serum and plasma (miRBase V18.0 nomenclature).

hsa-let-7a	hsa-miR-148a	hsa-miR-221	hsa-miR-338-3p
hsa-let-7b	hsa-miR-148b	hsa-miR-222	hsa-miR-339-3p
hsa-let-7b*	hsa-miR-150	hsa-miR-223	hsa-miR-342-3p
hsa-let-7d	hsa-miR-151-5p	hsa-miR-223*	hsa-miR-363
hsa-let-7d*	hsa-miR-152	hsa-miR-23a	hsa-miR-374b
hsa-let-7f	hsa-miR-15a	hsa-miR-23b	hsa-miR-375
hsa-let-7g	hsa-miR-15b	hsa-miR-24	hsa-miR-378
hsa-let-7i	hsa-miR-15b*	hsa-miR-25	hsa-miR-423-3p
hsa-miR-101	hsa-miR-16	hsa-miR-26b	hsa-miR-423-5p
hsa-miR-103	hsa-miR-16-2*	hsa-miR-27a	hsa-miR-424
hsa-miR-106a	hsa-miR-17	hsa-miR-27b	hsa-miR-425
hsa-miR-106b	hsa-miR-181a	hsa-miR-28-3p	hsa-miR-425*
hsa-miR-107	hsa-miR-185	hsa-miR-28-5p	hsa-miR-451
hsa-miR-122	hsa-miR-186	hsa-miR-29a	hsa-miR-484
hsa-miR-125a-5p	hsa-miR-18a	hsa-miR-29b	hsa-miR-486-5p
hsa-miR-125b	hsa-miR-18b	hsa-miR-29c	hsa-miR-495
hsa-miR-126	hsa-miR-191	hsa-miR-301a	hsa-miR-502-3p
hsa-miR-128	hsa-miR-192	hsa-miR-30b	hsa-miR-505
hsa-miR-130a	hsa-miR-195	hsa-miR-30c	hsa-miR-532-3p
hsa-miR-133a	hsa-miR-199a-3p	hsa-miR-30d	hsa-miR-532-5p
hsa-miR-139-5p	hsa-miR-199a-5p	hsa-miR-30e	hsa-miR-574-3p
hsa-miR-140-3p	hsa-miR-19a	hsa-miR-30e*	hsa-miR-584
hsa-miR-140-5p	hsa-miR-19b	hsa-miR-32	hsa-miR-629
hsa-miR-142-3p	hsa-miR-20a	hsa-miR-320a	hsa-miR-652
hsa-miR-142-5p	hsa-miR-20b	hsa-miR-320b	hsa-miR-92a
hsa-miR-143	hsa-miR-21	hsa-miR-324-3p	hsa-miR-93
hsa-miR-144	hsa-miR-210	hsa-miR-324-5p	hsa-miR-93*
hsa-miR-144*	hsa-miR-215	hsa-miR-328	hsa-miR-99a
hsa-miR-145	hsa-miR-22	hsa-miR-331-3p	hsa-miR-99b
hsa-miR-146a	hsa-miR-22*	hsa-miR-335	

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