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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.