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Comparison of RNA isolation and associated methods for extracellular RNA detection by high-throughput quantitative polymerase chain reaction

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression. miRNAs in biofluids are being used for clinical diagnosis as well as disease prediction. Efficient and reproducible isolation methods are crucial for extracellular RNA detection. To determine the best methodologies for miRNA detection from plasma, the performance of four RNA extraction kits, including an in-house kit, were determined with miScript miRNA assay technology; all were measured using a high-throughput quantitative polymerase chain reaction (qPCR) platform (BioMark System) with 90 human miRNA assays. In addition, the performances of complementary DNA (cDNA) and preamplification kits for TaqMan miRNA assays and miScript miRNA assays were compared using the same 90 miRNAs on the BioMark System. There were significant quantification cycle (Cq) value differences for the detection of miRNA targets between isolation kits. cDNA, preamplification, and qPCR performances were also varied. In summary, this study demonstrates differences among RNA isolation methods as measured by reverse transcription (RT)–qPCR. Importantly, differences were also noted in cDNA and preamplification performance using TaqMan and miScript. The in-house kit performed better than the other three kits. These findings demonstrate significant variability between isolation and detection methods for low-abundant miRNA detection from biofluids.

The discovery of microRNAs (miRNAs), small noncoding RNA molecules (containing ~22 nt) found in plants, animals, and some viruses, has altered our understanding of gene expression regulation [1]. miRNAs are found extracellularly in plasma, urine, cerebrospinal fluid, and saliva, and they are significantly stable in these biofluids [2–5]. Extracellular miRNAs may play an important role in cell-to-cell communication and other complex processes. In addition, the levels of miRNAs in biofluids have been associated with a wide variety of diseases [6–8] and have potential as biomarkers [4]. In this setting, these RNA molecules are detected within extracellular vesicles (EVs) such as exosomes, microvesicles, and oncosomes and are bound to proteins [9,10]. However, both isolation and analysis of cell free RNAs from biofluids present several new challenges. Isolation kits specifically designed to isolate RNAs from biofluids have been developed recently by several companies. Although many technologies measure miRNAs, reverse transcription (RT)–quantitative polymerase chain reaction (qPCR) remains the most sensitive and reproducible method [11]. High-throughput qPCR platforms are one of the best choices for reduction of cost and minimization of time for detection of broad miRNA signatures [12]. Optimization of methods for highly reproducible results is needed to use any measurement platform for disease characterization and accurate mechanistic description.

The first aim of this study was to compare the performances of three commercially available RNA isolation kits for biofluids and an In-House RNA Isolation Kit developed at the University of Massachusetts Medical School specifically for isolating miRNAs from human plasma samples [13]. The commercial kits were the

Abbreviations used: miRNA, microRNA; EV, extracellular vesicle; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; Cq, quantification cycle; CDF, cumulative distribution function; KS, Kolmogorov–Smirnov; SD, standard deviation; CV, coefficient of variation; mRNA, messenger RNA.

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miRCURY RNA Isolation Kit—Biofluids, the miRNeasy Serum/Plasma Kit, and the TaqMan miRNA ABC Purification Kit. The second aim was to compare two major RT–qPCR miRNA chemistries (miScript miRNA Assays from Qiagen and TaqMan miRNA Assays from Life Technologies) that are commercially available and can be used on the high throughput qPCR platform BioMark System (Fluidigm, South San Francisco, CA, USA). The same miRNA targets ($n = 90$) were used throughout the study.

Materials and methods

Blood sample collection and plasma and serum separation

For the study, 3 female and 3 male healthy volunteers on no medications were recruited. Written consent was obtained from the volunteers in accordance with the University of Massachusetts institutional review board. Blood samples were collected using standard venipuncture techniques into blood collection tubes with a liquid buffered sodium citrate additive (0.105 M) or serum tubes without anticoagulants. Citrated tubes were centrifuged at 2000*g* for 10 min at room temperature to collect plasma samples. Serum tubes were centrifuged at 2000*g* for 10 min at room temperature after a 30-min incubation at room temperature to allow clotting and acclimate to room temperature. Plasma and serum samples were then transferred into 2.0-ml microcentrifuge tubes and centrifuged again at 8000*g* for 10 min. The upper section of each plasma and serum sample was transferred into a clean 15-ml tube for pooling and careful mixing. Multiple aliquots (each 200 μl/tube) of plasma and serum pool samples were prepared in low DNA binding microcentrifuge tubes and stored at −80 °C until RNA isolation was performed.

RNA isolations from plasma and serum samples

RNA was isolated from pooled plasma and serum samples using three commercial kits—miRCURY RNA Isolation Kit—Biofluids (cat. no. 300112, Exiqon, Vedbaek, Denmark), miRNeasy Serum/Plasma Kit (cat. no. 217184, Qiagen, Frederick, MD, USA), and the TaqMan miRNA ABC Purification Kit (cat. no. 4473087, Life Technologies, Carlsbad, CA, USA)—and an In-House RNA Isolation Kit. The volume of both plasma and serum samples used for RNA isolations was kept constant at 200 μl for all isolation procedures. The manufacturers' protocols were followed with only minor changes; the recommended elution volumes were different for the various commercial kits and were adjusted as follows to be able to compare all kits. The miRNeasy Serum/Plasma Kit recommended 14 μl as the elution volume, and the volume was increased to 50 μl. The TaqMan miRNA ABC Purification Kit recommended 100 μl as the elution volume, and it was decreased to 50 μl. Life Technologies tech support confirmed that this change would not reduce the performance of this kit. The In-House Kit's RNA elution volume was 12 μl, and it was increased to 50 μl. The miRCURY RNA Isolation Kit—Biofluids did not require an adjustment in RNA elution volume. The eluted RNA samples were transferred into V-bottom, snap-cap, 0.5-ml micronic tubes in four aliquots (10 μl in each) and capped. Two-dimensional barcodes on the tubes were recorded, and RNA samples were stored and kept at −80 °C until needed.

The In-House Kit procedure was performed as follows. First, 200 μl of plasma samples was mixed with 100 μl of lysis buffer (6.4 M guanidine–HCl, 5% Triton, 5% Tween 20, 120 mM EDTA [ethylenediaminetetraacetic acid], and 120 mM Tris, pH 8.0) and 10 μl of Proteinase K (>600 mAU/ml, cat. no. 19131, Qiagen). This mixture was incubated at 60 °C for 15 min by shaking at 2000 rpm on an Eppendorf Thermomixer shaker. After the tubes were transferred to room temperature, 250 μl of nuclease-free water and 250 μl of phenol/chloroform 5:1 (pH 8.0) were added and mixed for 5 min at room temperature at 2000 rpm on a shaker. The tubes were centrifuged in a microcentrifuge tube at 16,000*g* for 5 min. Aqueous layers were collected and transferred into a 2.0-ml tube, and 1.5 ml of 100% ice-cold ethanol was added and mixed. This mixture was immediately transferred into an RNA Tini column (cat. no. EZC107, Enzymax, Lexington, KY, USA) and washed twice with 500 μl of ice-cold PE buffer (2 mM Tris [pH 7.5] and 20 mM NaCl in 80% ethanol). RNA Tini columns were spun at 16,000*g* for 2 min to dry the membrane. Then 50 μl of preheated (65 °C) nuclease-free water was pipetted onto the column membrane and spun at 16,000*g* for 2 min to elute RNA.

cDNA reactions

Two different commercial complementary DNA (cDNA) kits, miScript II RT Kit (cat. no. 218161, Qiagen) and TaqMan MicroRNA RT Kit (cat. no. 4366597, Life Technologies), were used in this study. The TaqMan MicroRNA RT Kit also required the use of Megaplex RT Primer Human Pool A from Megaplex Primer Pools, Human Pools Set version 3.0 (cat. no. 4444750, Life Technologies). TaqMan MicroRNA RT reaction conditions were 2 min at 16 °C, 1 min at 42 °C, 1 s at 50 °C total of 40 cycles, then hold 5 min at 85 °C. miScript II RT reaction conditions were 1 h at 37 °C and 5 min at 95 °C. All RT reactions were performed in a ProFlex PCR System (96-well block model) (Life Technologies). The following modifications were made in order to perform cDNA reactions in equal volumes in both kits. The TaqMan MicroRNA RT Kit reaction volume was 7.5 μl and reaction conditions were adjusted to a 10-μl reaction volume. In addition, 4 μl of RNA samples was used for all cDNA reactions. All cDNA samples were diluted 1:5 with nuclease-free water and then stored at −20 °C until preamplification reactions were performed.

Preamplification reactions

The miScript Microfluidics PreAMP Kit (cat. no. 331455, Qiagen) was used to preamplify the cDNAs made by the miScript II RT Kit. The TaqMan PreAmp Master Mix (cat. no. 4391128, Life Technologies) was used to preamplify the cDNAs made by the TaqMan MicroRNA RT Kit. The manufacturer's instructions were followed. Here, 5 μl of 1:5 diluted cDNA was used in all preamplification reactions. The final preamplification volume was 25 μl for both preamplification kits. Preamplified PCR products were diluted 1:5 with nuclease-free water and then stored at −20 °C until qPCRs were performed.

Dried down miScript miRNA assays were reconstituted to 100-μM concentrations to allow preamplification primer pool preparation. The miScript miRNA preamplification primer pool was prepared according to page 36 of the miScript Microfluidics Handbook (Qiagen). Megaplex PreAmp Primers Human Pool A from Megaplex Primer Pools, Human Pools Set version 3.0 (Life Technologies) was used with TaqMan PreAmp Master Mix. In total, 12 cycles of preamplification were performed with both kits.

qPCR analysis

In total, 90 human miRNA assays (Table 1) were selected based on the results of previous published plasma miRNA studies. miScript miRNA Assays (Qiagen) and TaqMan miRNA Assays (Life Technologies) were purchased. The 96.96 Dynamic Array Chip for Gene Expression (cat. no. BMK-M-96.96, Fluidigm, South San Francisco, CA, USA) was primed in the IFC Controller HX (cat. no. IFC-HX, Fluidigm).

miScript miRNA Assays (100 μM) were diluted to 40 μM and mixed with the same volume of miScript Microfluidics Universal