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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.

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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

Table 1
List of miRNA assays and miRBase accession numbers.

#	miRBase accession number	miRNA name	#	miRBase accession number	miRNA name	#	miRBase accession number	miRNA name
1	MIMAT0000684	hsa-miR-302a-3p	31	MIMAT0000461	hsa-miR-195-5p	61	MIMAT0000617	hsa-miR-200c-3p
2	MIMAT0000280	hsa-miR-223-3p	32	MIMAT0003885	hsa-miR-454-3p	62	MIMAT0000281	hsa-miR-224-5p
3	MIMAT0002854	hsa-miR-521	33	MIMAT0000083	hsa-miR-26b-5p	63	MIMAT0000438	hsa-miR-152-3p
4	MIMAT0000074	hsa-miR-19b-3p	34	MIMAT0003338	hsa-miR-660-5p	64	MIMAT0000419	hsa-miR-27b-3p
5	MIMAT0000069	hsa-miR-16-5p	35	MIMAT0000267	hsa-miR-210-3p	65	MIMAT0000099	hsa-miR-101-3p
6	MIMAT0002174	hsa-miR-484	36	MIMAT0000420	hsa-miR-30b-5p	66	MIMAT0000078	hsa-miR-23a-3p
7	MIMAT0001631	hsa-miR-451a	37	MIMAT0000680	hsa-miR-106b-5p	67	MIMAT0000763	hsa-miR-338-3p
8	MIMAT0002853	hsa-miR-519d	38	MIMAT0000082	hsa-miR-26a-5p	68	MIMAT0004810	hsa-miR-629-5p
9	MIMAT0000445	hsa-miR-126-3p	39	MIMAT0000250	hsa-miR-139-5p	69	MIMAT0000090	hsa-miR-32-5p
10	MIMAT0000080	hsa-miR-24-3p	40	MIMAT0004947	hsa-miR-885-5p	70	MIMAT0004775	hsa-miR-502-3p
11	MIMAT0000075	hsa-miR-20a-5p	41	MIMAT0000760	hsa-miR-331-3p	71	MIMAT0000094	hsa-miR-95-3p
12	MIMAT0000449	hsa-miR-146a-5p	42	MIMAT0004761	hsa-miR-483-5p	72	MIMAT0003161	hsa-miR-493-3p
13	MIMAT0000440	hsa-miR-191	43	MIMAT0003322	hsa-miR-652-3p	73	MIMAT0004920	hsa-miR-541-3p
14	MIMAT0000063	hsa-let-7b	44	MIMAT0000243	hsa-miR-148a-3p	74	MIMAT0003165	hsa-miR-545-3p
15	MIMAT0000279	hsa-miR-222	45	MIMAT0000722	hsa-miR-370-3p	75	MIMAT0001635	hsa-miR-452-5p
16	MIMAT0000451	hsa-miR-150	46	MIMAT0004748	hsa-miR-423-5p	76	MIMAT0003241	hsa-miR-576-5p
17	MIMAT0000092	hsa-miR-92a-3p	47	MIMAT0000272	hsa-miR-215-5p	77	MIMAT0003312	hsa-miR-642a-5p
18	MIMAT0000093	hsa-miR-93-5p	48	MIMAT0004552	hsa-miR-139-3p	78	MIMAT0004814	hsa-miR-654-3p
19	MIMAT0003393	hsa-miR-425-5p	49	MIMAT0000727	hsa-miR-374a-5p	79	MIMAT0003327	hsa-miR-449b-5p
20	MIMAT0000076	hsa-miR-21-5p	50	MIMAT0004780	hsa-miR-532-3p	80	MIMAT0003244	hsa-miR-579-3p
21	MIMAT0001413	hsa-miR-20b-5p	51	MIMAT0000068	hsa-miR-15a-5p	81	MIMAT0000432	hsa-miR-141-3p
22	MIMAT0000456	hsa-miR-186-5p	52	MIMAT0002872	hsa-miR-501-5p	82	MIMAT0003294	hsa-miR-625-5p
23	MIMAT0000421	hsa-miR-122-5p	53	MIMAT0001412	hsa-miR-18b-5p	83	MIMAT0002855	hsa-miR-520d-5p
24	MIMAT0002809	hsa-miR-146b-5p	54	MIMAT0000064	hsa-let-7c-5p	84	MIMAT0002171	hsa-miR-410-3p
25	MIMAT0000081	hsa-miR-25-3p	55	MIMAT0000446	hsa-miR-127-3p	85	MIMAT0000721	hsa-miR-369-3p
26	MIMAT0000227	hsa-miR-197-3p	56	MIMAT0000096	hsa-miR-98-5p	86	MIMAT0002834	hsa-miR-520a-3p
27	MIMAT0000244	hsa-miR-30c-5p	57	MIMAT0000688	hsa-miR-301a-3p	87	MIMAT0002890	hsa-miR-299-5p
28	MIMAT0000434	hsa-miR-142-3p	58	MIMAT0000720	hsa-miR-376c-3p	88	MIMAT0004774	hsa-miR-501-3p
29	MIMAT0003239	hsa-miR-574-3p	59	MIMAT0002175	hsa-miR-485-5p	89	MIMAT0001339	hsa-miR-422a
30	MIMAT0000431	hsa-miR-140-5p	60	MIMAT0004945	hsa-miR-744-5p	90	MIMAT0000271	hsa-miR-214-3p

Primer (40 μM). This mixture was mixed with an equal volume of 2× Assay Loading Reagent (cat. no. 85000736, Fluidigm). Then, 5 μl of this final mixture was pipetted into assay inlets of the 96.96 Dynamic Array Chip for Gene Expression.

TaqMan miRNA Assays (20×) were diluted with an equal volume of 2× Assay Loading Reagent, and 5 μl of this final mixture was pipetted into assay inlets of the 96.96 Dynamic Array Chip for Gene Expression.

Next, 3 μl of Microfluidics qPCR Master Mix from the miScript Microfluidic PCR Kit (cat. no. 331431, Qiagen) was mixed with 0.3 μl of 20× DNA Binding Dye Sample Loading Reagent (cat. no. 100-3738, Fluidigm), 0.7 μl of nuclease-free water, and 2 μl of diluted preamplified PCR products before 4 μl of this mixture was pipetted into sample inlets of the 96.96 Dynamic Array Chip for Gene Expression.

After 3 μl of TaqMan Universal Master Mix II (no uracil-*N*-glycoslyase) (cat. no. 4440047, Life Technologies) was mixed with 0.3 μl of 20× GE Sample Loading Reagent (cat. no. 100-7610, Fluidigm), 0.7 μl of nuclease-free water, and 2 μl of diluted preamplified PCR products, 4 μl of this mixture was pipetted into sample inlets of the 96.96 Dynamic Array Chip for Gene Expression.

The Dynamic Array Chip for Gene Expression was placed into the IFC Controller HX, and standard load script was started. After this process was completed (90 min), Dynamic Array Chips for Gene Expression were placed into the BioMark System and the thermal program was started for miScript miRNA Assays. The thermal program included 2 min at 50 °C, 30 min at 70 °C, and 10 min at 25 °C. A PCR initial activation step was performed at 95 °C for 10 min, then at 94 °C for 15 s (denaturation), at 55 °C for 30 s (annealing), and at 70 °C for 30 s (extension) with cycling for 40 cycles. In our analysis of qPCR results of miScript miRNA Assays, quantification cycle (Cq) values above 23 were marked as negative call based on the instructions for qPCR analysis on page 29 of the miScript Microfluidics Handbook. The thermal program used for TaqMan miRNA Assays was as follows: at 50 °C for 2 min, at 70 °C for 30 min, and at 25 °C for 10 min. After the PCR initial activation step was performed at 95 °C for 10 min, it was continued as 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing and extension as cycling for 30 cycles.

A single copy gives a Cq value of 26 or 27 by qPCR on the BioMark platform [14]. This Cq shift is due to miniaturized reaction volumes and density of florescent molecules in a tight chamber. A single copy Cq value is 36 in conventional plate-based qPCR platforms. In our analysis, Cq values above 27 were marked as a negative for TaqMan miRNA Assays. Sample and assay names were entered in Real Time PCR Analysis Software for the BioMark System. Linear derivative and user global settings (0.007) were used in the software for each miScript miRNA run, and linear derivative and auto global settings were used in the software as setting parameters for each TaqMan miRNA run. Then, data analyses were performed using BioMark Real Time PCR Analysis software to determine the Cq values.

Statistical analysis and result presentation

All statistical analysis, comparisons, and plots were made by R packages in R 3.2.0. Final results are presented in plots to allow easier interpretation and understanding of the obtained findings. Mainly, the data were represented in five different ways; box plots, cumulative distribution plots, density plots, heat maps, and all-to-all scatter plots including correlations between samples. Specifically for box plots and cumulative distribution plots, the ggplot2 0.9.3.1 package was employed. Average, standard deviation, and coefficient of variation values were used to calculate probability distributions in the cumulative distribution function (CDF). The CDF accumulates all of the probability less than or equal to x, where x is the certain Cq values. A general CDF is defined as the function $F(x) = P(X \leq x)$, where X is the random variable, which is the sum or

integral of the probability density function of the distribution. Density plots were made using the plotCtDensity function and heat maps were prepared using the plotCtHeatmap function in the HTqPCR 1.22.0 package. With all-to-all scatter plots, replicate and kit comparisons were demonstrated with their Spearman correlations. In the analysis, all No Amplification values were set to the maximum detected value of 26.

In addition to these, the average Cq distributions among different isolation kits were tested with the two-sample Kolmogorov–Smirnov (KS) test to show the significance of each isolation kit. The KS test is a nonparametric test used to compare two-sample distributions. The KS statistic is based on quantifying a distance between the empirical distributions of functions of two samples. The null hypothesis is that the samples are drawn from the same continuous distribution [15]. To perform the KS test, we used ks.test in R, and the results are shown in Supplemental Tables S1A and S1B in the online supplementary material [16].

Results

Bioanalyzer results of isolated RNA

To determine the performances of the isolation kits, RNA was isolated using the three commercial kits and an in-house isolation kit from pooled plasma samples according to the plan explained in Fig. 1A. RNA was analyzed on a 2100 Bioanalyzer instrument using the Agilent RNA 6000 Pico Kit (cat. no. 5067-1513, Agilent Technologies, Santa Clara, CA, USA), which focuses on 25- to 4000-nt RNAs. In addition, the Agilent Small RNA Kit (cat. no. 5067-1548, Agilent Technologies), which focuses on 4- to 150-nt RNAs, was used. The Agilent Small RNA Kit was unable to detect RNA from 200 μl of plasma, but the Agilent RNA 6000 Pico Kit was able to successfully detect and quantify the RNA. The RNA peak in the Pico kit was within the 25- to 200-nt band, but no discrete peaks were detected using the Small RNA kit. This is likely because the Small RNA kit focuses on a much smaller area (4–150 nt) than the Pico kit (25–400 nt), so in the Small RNA kit an array of RNA fragments that are of different sizes yet similar low abundance became spread across that range (hence no discrete peaks), whereas in the Pico kit, with its larger range, the RNA fragments were visualized more closely together on the *x*-axis and became detectable as a peak (see Supplemental Fig. 17A in supplementary material). The yields for the specific isolation kits are shown in Supplemental Fig. 17B. The highest yield was obtained with the miRNeasy Serum/Plasma Kit (9.20 ± 3.12 ng), whereas the miRCURY RNA Isolation Kit–Biofluids, In-House Kit, and TaqMan miRNA ABC Purification Kit, yielded 7.36 ± 2.07, 1.06 ± 0.44, 0.70 ± 0.26 ng of RNA, respectively (Fig. 2A).

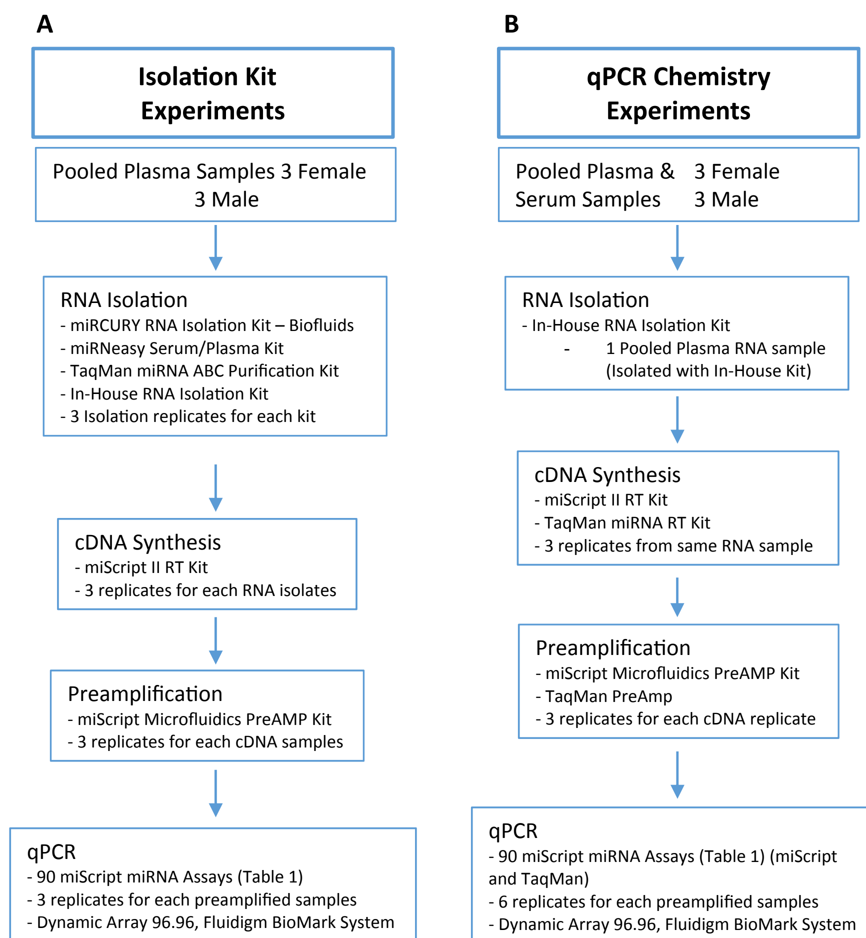
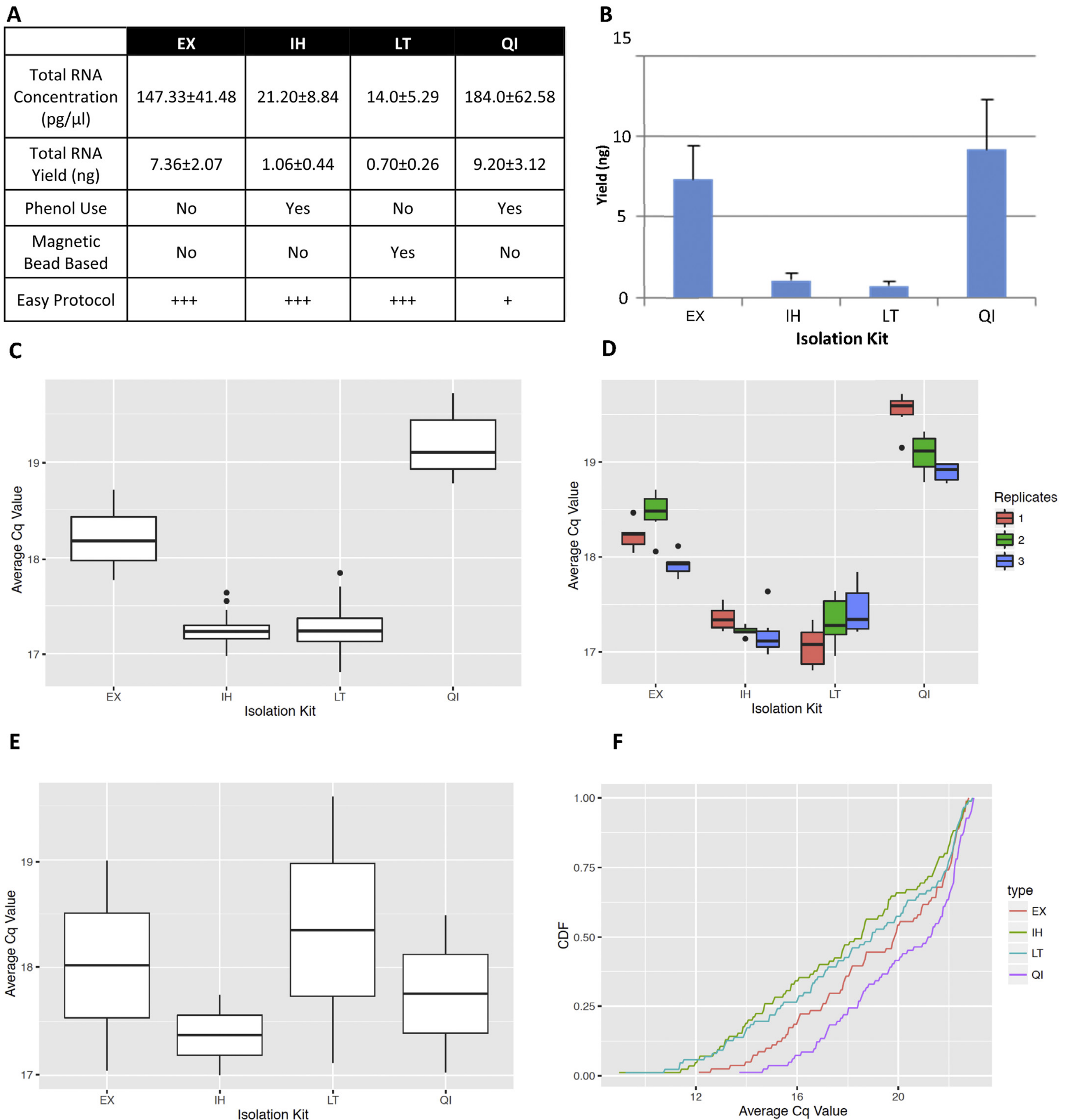


Fig.1. Experimental design and layout. (A) RNA isolation kit experiments. Plasma pool from 3 female and 3 male individuals was prepared. Three isolations were performed with each isolation kit. Each isolate was converted to cDNA in three replicates by using the miScript II RT kit. Each cDNA replicate was preamplified with the miScript Microfluidics PreAMP Kit three times. All preamplified samples were used in qPCR analysis three times for 90 miScript miRNA Assays. (B) qPCR chemistry experiments. RNA samples (from pooled plasma and serum samples) were converted to cDNA in triplicates. Each cDNA replicate was preamplified three times and each preamplified sample was used in qPCR analysis six times for 90 miScript miRNA Assays.



A

	EX	IH	LT	QI
Total RNA Concentration (pg/µl)	147.33±41.48	21.20±8.84	14.0±5.29	184.0±62.58
Total RNA Yield (ng)	7.36±2.07	1.06±0.44	0.70±0.26	9.20±3.12
Phenol Use	No	Yes	No	Yes
Magnetic Bead Based	No	No	Yes	No
Easy Protocol	+++	+++	+++	+

Fig.2. (A) RNA concentration and yield from each isolation kit: miRCURY RNA Isolation Kit—Biofluids (EX), In-House Isolation Kit (IH), TaqMan miRNA ABC Purification Kit (LT), and miRNeasy Serum/Plasma Kit (QI). (B) RNA yields from all isolation kits. (C) Performance of cDNA replicates for each RNA isolate. (D) Performance of preamplification replicates for each RNA isolate. (E) Coefficient of variation results for each RNA isolate (E). (F) CDF value for each RNA isolation kit.

RNA isolation kit, cDNA, and preamplification performances

The experimental plan is explained in Fig. 1A and B. The RNA isolation quality and quantity obtained using each isolation kit were tested after conversion to cDNA, preamplification, and qPCR. After cDNA reactions were performed in triplicate using the miScript II RT Kit, each cDNA sample was preamplified in triplicates by using the miScript Microfluidics PreAMP Kit. Then, all samples were analyzed for 90 miScript miRNA Assays using the BioMark System with a Dynamic Array Chip for Gene Expression in six replicates (Fig. 1B). All starting volumes for plasma, RNA, cDNA, preamplification, and qPCR were kept constant for all RNA isolation methods. This allowed for the analysis of Cq values for all isolation methods without any additional normalization.

The mean of nine total replicates for each cDNA replicate is shown in Fig. 2E. The lowest mean Cq values (mean of all miRNA assays Cq = 17.25) were obtained using the In-House Kit. In-House RNA samples also exhibit the lowest standard deviation (SD) values (mean of all miRNA assays SD = 0.41) between each isolation replicate. The next best performance was from the TaqMan miRNA ABC Purification Kit, where samples had a mean Cq and a mean standard deviation of 17.27 and 1.135, respectively. The respective mean Cq and mean SD were determined as 18.20 and 0.955 for the miRCURY RNA Isolation Kit–Biofluids and 19.17 and 0.73 for the miRNeasy Serum/Plasma Kit. As mentioned above, each cDNA sample was preamplified in triplicate, and each preamplification also was run in triplicate by qPCR on a Dynamic Array. The mean value of three qPCR runs from each preamplification replicate obtained using each isolation kit is shown in Fig. 2F. The In-House Kit and TaqMan miRNA ABC Purification Kit had superior performance as compared with the other isolation kits. The SD values for the In-House Kit were found to be superior to those for the TaqMan miRNA ABC Purification Kit. The mean coefficient of variation (CV) values for all qPCR replicates for all 90 miRNA assays were calculated as 1.86, 3.77, 5.10, and 6.75% for the In-House Kit, miRNeasy Serum/Plasma Kit, miRCURY RNA Isolation Kit–Biofluids, and TaqMan miRNA ABC Purification Kit, respectively (Fig. 2E). CDF analysis results are shown in Fig. 2F. The lowest cumulative distribution of Cq values were found with the In-House Kit. The highest cumulative distribution was found with the miRNeasy Serum/Plasma Kit.

Correlation values for the isolation kit replicates were among 0.93, 0.99, 0.94%; 0.99, 0.98, 0.97%; 0.97, 0.97, 0.99%; and 0.95, 0.96, 0.97% for the In-House Kit, TaqMan miRNA ABC Purification Kit, miRCURY RNA Isolation Kit–Biofluids, and miRNeasy Serum/Plasma Kit, respectively (see Supplemental Fig. 1 in supplementary material). Delta Cq values of all RNA isolates for miR-23a to miR-451 were found to be lower than 5, which is consistent with a lack of red blood cell hemolysis in the plasma samples (data not shown) [17].

Comparison of miScript miRNA and TaqMan miRNA Assays

The same set of 90 miRNAs (90 TaqMan miRNA Assays and miScript miRNA Assays for the same 90 miRNAs) were used to determine the performance of TaqMan and miScript miRNA chemistries. The RNA samples were isolated from the pooled plasma and pooled serum samples using the In-House Kit. The RNA samples were split into two aliquots. Each aliquot was used for both the miScript II RT Kit and TaqMan MicroRNA RT Kit. All plasma and serum pool samples were converted to cDNA in triplicate for each cDNA kit to determine cDNA variability with each technology. Detailed cDNA, preamplification, and qPCR replicates and the experimental plan and layout are explained in Fig. 1.

cDNA kit performance

The miScript II RT Kit had superior performance to the TaqMan MicroRNA RT Kit. The Cq values for serum RNA samples were lower for both technologies as compared with the plasma RNA Cq values. The mean Cq values for all cDNA replicates were 19.29 and 18.05 for plasma RNA and serum RNA, respectively, using the miScript II RT Kit and were 22.40 and 21.25 for plasma RNA and serum RNA, respectively, using the TaqMan MicroRNA RT Kit. In addition, SD values were determined to be lower (Fig. 3A).

Preamplification kit performances

The miScript Microfluidics PreAMP Kit was superior to the TaqMan PreAmp Master Mix. The mean Cq values for the miScript Microfluidics PreAMP Kit had a range between 19.11 and 19.51 for plasma samples and between 18.01 and 18.11 for serum samples. The mean Cq values for the TaqMan PreAmp Master Mix ranged between 22.17 and 22.63 for plasma samples and between 20.82 and 21.58 for serum samples (Fig. 3B).

Correlations between replicates in each miRNA technology

It was determined that the correlation values varied between 0.31 and 0.94 for TaqMan miRNA Assay replicates and between 0.82 and 0.97 for miScript miRNA Assay replicates in plasma pool samples (Fig. 4A and C). The values were found to be between 0.77 and 0.97 for TaqMan miRNA Assay replicates and between 0.97 and 0.99 for miScript miRNA Assay replicates in serum pool samples (Fig. 4B and D).

Cq distributions for miScript and TaqMan chemistries

miScript miRNA Assay and TaqMan miRNA Assay Cq distributions varied. The miScript miRNA Assay Cq distribution was between 6 and 27 for plasma pool samples and between 5 and 27 for serum samples. The TaqMan miRNA Assay Cq distribution was between 14 and 27 for plasma pool samples and between 7 and 27 for serum samples (Fig. 3C).

Discussion

In this study, we found that the In-House RNA Isolation Kit performed best among the four isolation kits that we compared, with the lowest Cq values and lowest SD values. We also found that the reproducibility of the miScript miRNA Assay technology was superior to that of the TaqMan miRNA Assay technology on the high-throughput BioMark platform. The results of this study provide the first comparison of two widely used qPCR chemistries using the BioMark System, a high-throughput qPCR platform, with a large number of human miRNA assays. There are published studies that are similar, but none of them analyzed this many miRNA assays at once. This is also the first study to compare different RNA isolation kits specifically designed for biofluids by analyzing 90 human miRNA assays and high-throughput qPCR. An earlier study analyzed a wider variety of isolation kits but did not analyze their performances using a large group of miRNAs by RT–qPCR [18]. Mestdagh and coworkers analyzed a large number of miRNA expression platforms for serum samples and control samples as well as other biological samples; however, they used various sequencing, microarray, and qPCR platforms but not a high-throughput qPCR platform [11]. Redshaw and coworkers compared the performances of TaqMan miRNA Assays and miRCURY LNA miRNA Assays using a 7900 HT system with synthetic RNA molecules; however, they did not include miScript miRNA Assays [19]. Another study compared mirVana, miRCURY Cell and Plant, and TRIzol LS kits, miRCURY Biofluids Kit, and miRNeasy Plasma/Serum Kit from Qiagen with plasma and cerebrospinal fluid [20]. In another study, Tan and coworkers analyzed limited numbers of human miRNAs ($n = 16$) in human plasma samples using the BioMark System. They also found that the variability for each kit as measured by the use of synthetic spike-ins was high [21]. Brunet-Vega and coworkers compared five commercially available kits for serum/plasma miRNA isolation. They found that all isolation methods were suitable for extracting miRNA from plasma samples and had similar Cq values [22].

The manufacturers of the miRNeasy Serum/Plasma Kit, miRCURY RNA Isolation Kit–Biofluids, and TaqMan miRNA ABC Purification Kit state that these methods are designed for biofluids. The In-House Kit was also designed for RNA isolation from biofluids. Both the miRCURY RNA Isolation Kit–Biofluids and TaqMan miRNA

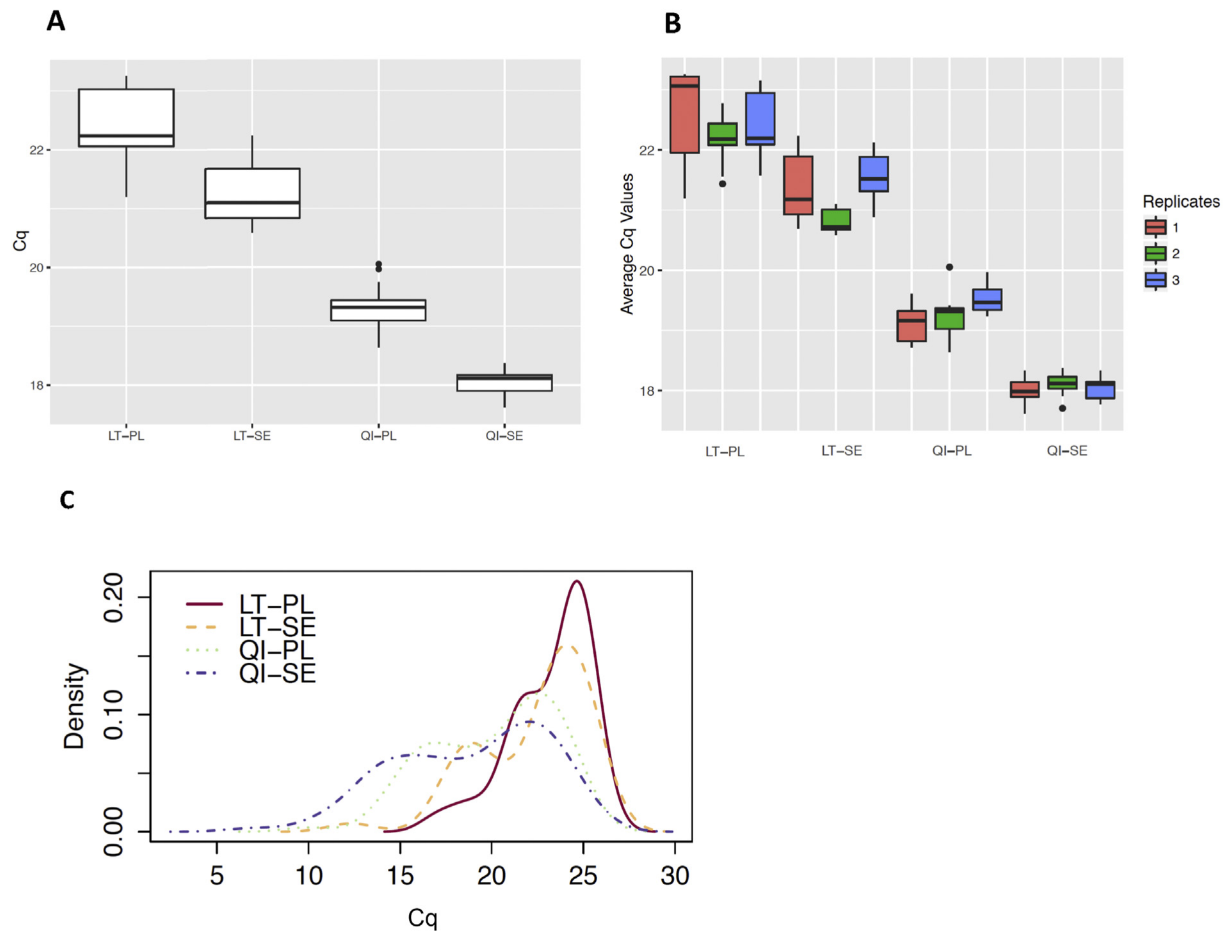


Fig.3. (A) Plasma (PL) and serum (SE) RNA sample cDNA replicate performance for Life Technologies TaqMan Assay technology (LT) and Qiagen miScript miRNA technology (QI). (B) Plasma and serum RNA sample preamplification replicate performance for Life Technologies TaqMan Assay technology and miScript miRNA technology. (C) Cq distribution of plasma and serum RNA samples for both TaqMan and miScript miRNA technologies.

ABC Purification Kit do not require phenol. The TaqMan miRNA ABC Purification Kit manufacturer states that it is only designed to isolate miRNAs within the company's panel A and B miRNA lists. However, we chose to include this kit in our study because it is the only non-column magnetic bead preparation. The miRCURY RNA Isolation Kit—Biofluids protocol required the shortest experimental time.

We found that the In-House Isolation Kit was superior to the commercially available kits tested based on lower observed Cq values consistent with higher sensitivity, potentially better detection of very low-abundant miRNA targets in biofluids, lower SD values, and higher reproducibility. This kit was also the least expensive of those tested at less than $2 (U.S.) per sample. The use of Tini columns by Enzymax with the In-House Kit allows for the elution of RNA in much smaller volumes (as low as 6 μl) to concentrate the eluted RNA for RT reactions. The miRNeasy Serum/Plasma Kit has a difficult aqueous phase separation, potentially creating differences between samples, given that there is no assurance that the same volume of aqueous phase separation will be obtained from each sample.

Li and coworkers demonstrated different isolation kits having broad ranges of RNA yield and differences in their detection of miRNAs [18]. We believe that using a large number of miRNA assays to evaluate the performances of isolation kits and quantitative analysis chemistries is crucial to detect optimal methods. Possible bias based on sequences of different miRNAs during the isolation process and RT–qPCR steps can be understood only by analyzing large numbers of miRNAs.

In our experimental design, we kept the volumes for all reactions, such as input RNA, cDNA, and preamplification, constant between each of the chemistries. This allowed us to compare both technologies without applying housekeeping normalization. This approach was employed because of the current lack of consensus in regard to the use of housekeeping miRNAs for plasma and serum given that even using housekeeping miRNAs to normalize data is not as clear as the direct comparison by keeping variables (volumes) constant. Lower Cq values and SD values in serum RNA samples compared with plasma RNA samples is probably due to the anticoagulant dilution effect in plasma samples. The main purpose of running serum samples in this study was not to compare serum with plasma; rather, we used serum as a form of control that did not have any additions in blood samples such as any anticoagulant.

High-throughput qPCR platforms are especially useful for rapidly profiling large numbers of miRNAs from many samples. Unlike messenger RNAs (mRNAs) (~23,000), there is a limited number of miRNAs (~2500). Although it is not possible to analyze all mRNAs for large numbers of samples with these high-throughput platforms, it is possible to analyze large numbers of samples for all miRNAs. The high-throughput BioMark System is a flexible high-throughput qPCR platform allowing for changes of chemistry and assays as needed.

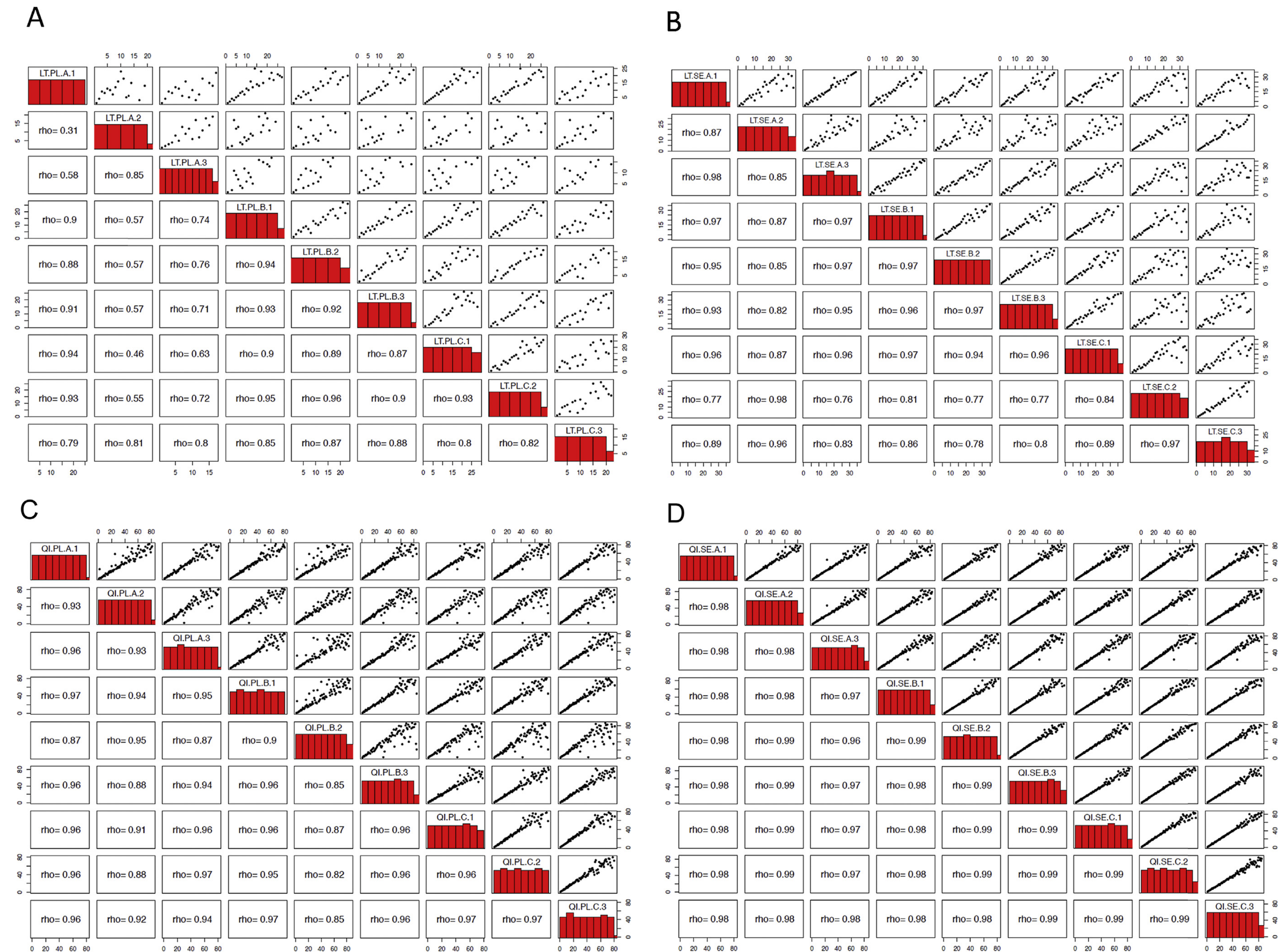


Fig.4. (A, B) Correlations for TaqMan technology replicates for plasma RNA samples (A) and serum RNA samples (B). (C, D) Correlations for miScript technology replicates for plasma RNA samples (C) and serum RNA samples (D). LT, TaqMan assay technology; PL, plasma RNA sample; IH, In-House isolation method; A, B, and C, cDNA replicates from same RNA samples; 1, 2, and 3, preamplification replicates from each cDNA sample and each preamplification sample run on qPCR in six replicates.

Conclusions

This study demonstrates the comparative performance of two widely used miRNA RT–qPCR chemistries on a high-throughput qPCR platform by analyzing large numbers of human miRNA assays. The results of the study suggest that, in this setting, a newly developed in-house RNA isolation kit for biofluids was the most reliable. The findings also suggest that combining highly sensitive RNA isolation kits and reproducible miRNA assay chemistry using a high-throughput qPCR platform is extremely valuable for larger studies analyzing circulating miRNAs from human plasma and serum.

Author contributions

J.E.F. and K.T. coordinated the project's laboratory studies and writing of laboratory methods. A.K. analyzed the results. E.M. and S.E.T. conducted experiments. R.L. and V.R.A. developed the in-house RNA isolation kit. All authors commented on the interpretation of results and also reviewed and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.02.019.

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