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Analytics of Cerebrospinal Fluid MicroRNA Quantitative PCR Studies

Theresa A. Lusardi[1] · Jack T. Wiedrick[2] · Molly Malone[3] · Jay I. Phillips[3] · Ursula S. Sandau[3] · Babett Lind[4] · Joseph F. Quinn[4,5] · Jodi A. Lapidus[2] · Julie A. Saugstad[3]

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression. Recent studies have shown that human disease states correlate with measurable differences in the level of circulating miRNAs relative to healthy controls. Thus, there is great interest in developing clinical miRNA assays as diagnostic or prognostic biomarkers for diseases, and as surrogate measures for therapeutic outcomes. Our studies have focused on miRNAs in human cerebral spinal fluid (CSF) as biomarkers for central nervous system (CNS) diseases. Our objective here was to examine factors that may affect the outcome of quantitative PCR (qPCR) studies on CSF miRNAs, in order to guide planning and interpretation of future CSF miRNA TaqMan® low-density array (TLDA) studies. We obtained CSF from neurologically normal (control) donors and used TLDAs to measure miRNA expression. We examined sources of error in the TLDA outcomes due to (1) nonspecific amplification of products in total RNA, (2) variations in RNA isolations performed on different days, (3) miRNA primer probe efficiency, and (4) variations in individual TLDA cards. We also examined the utility of card-to-card TLDA corrections and use of an unchanged "reference standard" to remove batch processing effects in large-scale studies.

Keywords Cerebrospinal fluid · microRNA · Quantitative PCR · TaqMan low-density array · Analytics

Introduction

In medicine, a biomarker is defined as "a specific physical trait used to measure or indicate the effects or progress of a disease, illness, or condition" [1]. Extracellular RNAs (exRNAs) in biofluids offer great promise as diagnostic and/or prognostic biomarkers for human diseases [2]. While many biofluids (e.g., blood, urine, and saliva) can be obtained using minimally invasive procedures, cerebral spinal fluid (CSF) can only be obtained through more invasive procedures such as a lumbar puncture. Yet CSF is the most informative biofluid regarding changes that occur in central nervous system (CNS), and CSF biomarkers have been identified for a number of diverse neurological diseases [3] including Alzheimer's disease (AD) [4–7], malignancies [8, 9], and psychiatric diseases [10]. CSF exRNAs include microRNAs (miRNAs) that are relatively stable in biofluids, presumably due to protection afforded by their containment in extracellular vesicles or their association with lipoprotein particle complexes. We used TaqMan® low-density array (TLDA) to quantify miRNA expression in lumbar CSF obtained from living donors, and identified a set of miRNAs as candidate biomarkers for AD [11]. Our goal here was to examine potential sources of measurement error that may occur in the course of large-scale CSF miRNA expression studies using TLDAs, in order to guide the planning and interpretation of future qPCR studies in CSF.

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✉ Julie A. Saugstad
saugstad@ohsu.edu

[1] Cancer Early Detection Advanced Research Center, Knight Cancer Institute, Oregon Health & Science University, Portland, OR, USA

[2] Biostatistics & Design Program, Oregon Health & Science University, Portland, OR, USA

[3] Department of Anesthesiology & Perioperative Medicine, Oregon Health & Science University, Portland, OR, USA

[4] Department of Neurology, Layton Aging and Alzheimer's Center, Oregon Health & Science University, Portland, OR, USA

[5] Portland VA Medical Center, Portland, OR, USA

Materials and Methods

Experimental Design

Table 1 shows the experimental design for the studies and outcomes presented here, including the goal of each study,

number of CSF samples used for RNA isolation, treatment of RNA (if any), the quantity of TLDA cards used for miRNA quantification, and the corresponding figure(s) for each experiment. Experiments 1–4 were performed using aliquots from a single neurologically normal CSF donor. Experiment 5 was performed using a pool of CSF from two neurologically normal control donors for the reference standard (RefStd) and normal control CSF donors for the card alignment.

Ethics Statement

CSF was obtained from neurologically normal, control donors who provided written informed consent. The Institutional Review Board of Oregon Health & Science University (OHSU) approved all procedures performed at the Oregon Alzheimer's Disease Center (OADC) under IRB 6845. Participants undergo detailed clinical and laboratory evaluation. CSF samples are banked at the OADC, the core program of the Layton Aging & Alzheimer's Disease Center, supported by the National Institute on Aging.

CSF Collection

The OADC has a standardized CSF collection protocol corresponding to that used in other AD centers engaged in CSF biomarker research [12]. All CSF collections are done in the AM under fasting conditions, in the lateral decubitus position, with a 24-gauge Sprotte spinal needle that minimizes discomfort of the procedure and reduces the incidence of lumbar puncture headache. The first 3 mL of CSF are discarded to reduce the possibility of blood or lidocaine contamination. Then, the next 2–3 mL are sent to a OHSU clinical lab to test for amounts of red blood cells, white blood cells, protein, and glucose. Subsequent serial syringes of 10–20 mL of collected CSF are mixed and transferred to polypropylene tubes that include a subject number, but no other identifying information. CSF is aliquoted into 0.5 mL tubes, flash frozen on dry ice, and stored at − 80 °C. For experiments 1–4, we used 20 mL of CSF from a normal, control donor, and all lab tests for this donor were in normal range [clear appearance, colorless, WBC count of 2 cu mm (range 0–5/cu mm), RBC count of < 1 cu mm, glucose 49 mg/dL (range 40–70 mg/dL), and protein 38 mg/dL (range 15–45 mg/dL)].

RNA Isolation and Amplification

Total RNA (not enriched for small RNAs) was isolated from 0.5 mL CSF using the mirVana™ PARIS™ RNA and Native Protein Purification Kit (ThermoFisher Scientific), with modifications [13]. RNA samples were concentrated (RNA Clean & Concentrator™-5 Kit; Zymo Research) and eluted into 8 μL of RNAse/DNase-free water. Then, 3.2 μL of concentrated RNA was transcribed using MultiScribe™ Reverse Transcriptase (ThermoFisher Scientific) in an 8 μL reaction volume, and 2.5 μL of the cDNA was preamplified in separate reactions with Megaplex™ RT Primer Pool Set v3.0.A or v3.0.B on a T-100 thermocycler (Bio-Rad, Hercules, CA) following the manufacturer's instructions for detection of miRNAs with preamplification. The preamplification products were diluted into a prescribed final volume of 100 μL with water, and stored at − 20 °C.

DNase and RNase Treatment of Total RNA

Total RNA from three CSF aliquots was pooled to ensure that the input RNA was identical in each group, then the RNA was separated into four groups: group 1 had no treatment; group 2 was treated with RNase-Free DNase 1 at 37 °C for 2 h (OPTIZYME™ DNase I, Fisher BioReagents™, Cat. No. BP8107-1); group 3 was treated with RiboShredder™ RNase Blend at 37 °C for 2 h (Epicentre, Madison, WI; Cat. No. RS12100); and group 4 had reverse transcriptase omitted from the reactions. The RNAse and DNase were removed from the RNA by acid phenol treatment, and then concentrated using Zymo columns. Reverse transcription and preamplification of groups 1–3 were performed according to our standard protocol [11]. For group 4, water was used in place of reverse transcriptase enzyme, with all other conditions following the standard protocol. For the no template control (NTC) assay, water was used in place of total RNA in the reaction.

MicroRNA Arrays

The CSF miRNAs were measured using TaqMan® Array Human MicroRNA Card Set v3.0, a two-card set (A + B) containing a total of 754 miRNA assays, plus probes for U6 snRNA (U6), RNU44, RNU48, and ath-miR-159a (spike-in control) (ThermoFisher, catalog # 4444913). Eighteen of the 754 miRNAs were excluded from analysis based on status updates in miRBase (www.mirbase.org). The qPCR amplifications were done on a QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher) and data acquired using automated baseline and threshold values determined by Expression Suite™ software v1.2.2 (ThermoFisher). We acknowledge that MIQE guidelines [14] propose the use of quantification cycle (Cq) vs threshold cycle (Ct). However, note that we use Ct for consistency with the Expression Suite software qPCR data reports, and Cq for the Expression Suite quality flags presented in the data. These include the amplification score (AmpScore) which indicates that, for a given well, the amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings, and the Cq confidence score (CqConf) which indicates that the calculated confidence for the Ct value of the well is less than the minimum value defined in the analysis settings.

Table 1 Experimental design. The table lists each experimental goal, the protocol and number of TLDA card sets, and the figures for each experiment

Experiment	Protocol	Figure
Non-specific amplifications in total RNA	3 Aliquots of pooled control CSF 3 Individual RNA isolations	1a, b
	4 Treatments for each RNA isolate (none, DNase, RNase, no RT)	
	12 TLDA card sets	
Effect of individual RNA isolations on qPCR outcomes	10 Aliquots of pooled control CSF 10 Individual RNA isolations	2a, b
	10 TLDA card sets	
Efficiency of miRNA primer probes	15 Aliquots of pooled control CSF 15 Individual RNA isolations	3
	RNA concentrated and pooled	
	Serial dilution (0.1X, 0.3X, 1X, 3X)	
	16 TLDA card sets	
Effect of individual TLDA cards on qPCR outcomes	10 Aliquots of pooled control CSF 10 Individual RNA isolations	4a, b
	RNA pooled	
	10 TLDA card sets	
Reference standard correction for card-to-card TLDA variation	Individual control CSF samples	5a, b

Filtering of the Ct values consisted of (i) qPCR products were considered below the detection threshold and censored if Ct ≥ 40 or if Expression Suite reported the Ct value as "Undetected"; (ii) individual assays were excluded if AmpScore < 1.0. All other Cts were accepted as reported by Expression Suite.

Data Centering and Normalization

Data centering was performed using CSF miRNA Ct values from an unchanged reference standard (RefStd) sample that was intercalated into a study that took place over several weeks. The RefStd was generated by pooling 20 mL of CSF from two individual control donors, aliquoting the 40 mL of CSF into 0.5 mL tubes, and storing at − 80 °C. We then incorporated one aliquot of the RefStd into each batch of RNA isolations done on one date. To correct for batch effects across the study, each card was centered within a batch by lining up the RefStd medians. The median of all RefStd Ct values within a batch was compared to the overall RefStd median (of all the within-batch medians), and the difference was used as a batch correction: (within-batch RefStd median) − (cross-batch median of within-batch RefStd medians). We centered the per-card values within a sample using the same idea, comparing each within-card U6 median to the mean of the two within-card U6 medians, using the difference as a card correction for the sample: (within-card U6 median) − (cross-card mean of within-card U6 medians). Ct values were then normalized across all samples by the (now centered and batch-corrected) within-sample U6 levels. The normalized values were calculated for each well as (batch-corrected and centered Ct) − (batch-corrected and centered U6 Ct) + (grand median of pre-centered Ct values). The effect of this normalization is absolute alignment of median U6 values across all samples, so that Ct distances are all on the same scale, anchored at U6.

Results

Test for Nonspecific Amplification of Products in Total RNA

We examined whether nonspecific amplification of products in human CSF total RNA effect miRNA measurements. We compared miRNA expression in total RNA from four groups: (1) No treatment, (2) DNase treated, (3) RNase treated, or (4) no RT enzyme in the reverse transcription reaction. Table 2 lists the number of amplified products that are detected at a Ct cutoff of 34 (Table 2(A)) or 40 (Table 2(B)). Good = miRNAs that performed well in the amplifications; censored = miRNAs either not present in the sample, or below the detection threshold in the TLDA assay; excluded = miRNAs that failed for technical reasons; questionable = miRNAs with a low Cq confidence value. The number of successful amplifications (of 0–3 possible) for each miRNA in each group is listed in Supplemental Table D-G. The no treatment group yielded an average of 125 "Good" qPCR products on the arrays at Ct < 34, while the DNase group had an average of number of 99 products, or 21% of the total "Good" amplifications (Table 2(A)). The RNase group had an average of 54 products, a 57% reduction from the no treatment group, while the no RT enzyme group had an average of 21 products, a reduction of

Table 2 Number and categories of qPCR products. The table lists those qPCR products detected at a cutoff at a Ct of 34 (A) or 40 (B) for $n = 3$ samples within each treatment group. The number of qPCR products is subdivided into categories designated as good, censored, excluded, or questionable

	No treatment			DNase			RNase			No RT enzyme		
A. Ct cutoff = 34												
Isolate	1	2	3	1	2	3	1	2	3	1	2	3
Good	123	121	130	82	103	111	51	54	56	22	19	22
Censored	555	539	524	611	545	543	639	618	612	716	725	720
Excluded	77	101	102	72	113	100	75	93	93	28	23	24
Questionable	13	7	12	3	7	14	3	3	7	2	1	2
Total	768	768	768	768	768	768	768	768	768	768	768	768
B. Ct cutoff = 40												
Isolate	1	2	3	1	2	3	1	2	3	1	2	3
Good	127	123	132	85	107	112	53	56	58	22	20	25
Censored	507	486	471	570	504	503	593	569	556	690	704	700
Excluded	119	150	151	108	149	138	119	138	146	54	43	41
Questionable	15	9	14	5	8	15	3	5	8	2	1	2
Total	768	768	768	768	768	768	768	768	768	768	768	768

Censored: (Ct = undetermined) or (AmpScore = 0) or (CqConf = 0) or Cq ≥ 40 (A) or 34 (B) Excluded: (not censored) and (AmpScore < 1.0); questionable: CqConf < 0.9

83% of amplified products. Figure 1**a** shows correlating histograms (frequency of amplifications vs Ct) of the products in each group. Note that DNase reduced the number of products relative to the no treatment group, particularly for products at Ct values < 22, while in the RNase most but not all of the products in this Ct range are eliminated. Supplemental Table H lists the Ct values for 35 products (34 miRNAs plus U6; $n = 1$ technical replicate) that amplified in the no template control (NTC). Many of the miRNAs that amplified in the NTC were also detected in the DNase and RNase treatment groups (D–G). Figure 1b shows correlation plots for each group: the left graphical boxes are miRNA correlation plots for each respective comparison, the right boxes are correlation R^2 values. Axis labels are raw Ct values. The plot shows a strong correlation between products in the no treatment and DNase-treated samples, but not between the no treatment and the RNase-treated or no RT enzyme groups. These results show that most products amplified from CSF total RNA in these arrays are miRNAs. However, there are a small number of additional products, likely the result of contaminating DNA in the CSF, or RNAse-resistant RNAs.

Effect of Individual RNA Isolations on qPCR Outcomes

We examined variations in outcomes that may occur due to the isolation of RNA batches on separate days. We isolated total RNA from five aliquots of 0.5 mL CSF on one date, then repeated the isolation protocol on a second later date, then performed the RT and amplification reactions at the same time. We calculated the mean Ct value, the standard deviation (SD), the number of arrays that the miRNA was detected in (Count), the coefficient of variation (CoV), and the lower and upper confidence interval (CI) for each miRNA detected on the arrays (Supplemental Table I–N). A correlation plot for the outcomes of the five RNA samples isolated on the first date (group 1: 1.1–1.5) and the second date (group 2: 2.1–2.5) is shown in Fig. 2a. The plot shows a high correlation between outcomes from four of the five RNA isolates in group 1. The high variability of RNA isolate 1.1 correlated with technical failure of the TLDA B card, identified by failure to amplify U6, and this sample would be excluded from further analysis. The plot also shows a high correlation between outcomes from all of the RNA isolates in group 2. However, the correlation is reduced between groups 1 and 2, suggesting that RNA isolation on different dates can affect outcomes. We considered a card-wise normalization approach to increase the group-level concordance, in which the within-card median Ct value was subtracted from each individual Ct value. We calculated a Pearson correlation for group 1 vs group 2 (Fig. 2b)**.** In the raw Ct plot (left), we calculated a 0.789 correlation between the two groups. In the corrected Ct plot (right), we calculated a 0.821 correlation between the two groups. Also note that the concordance is improved, in that the points now cluster more closely to the diagonal line of agreement. While this appears to be a modest improvement for $n =$ ten samples (five/group), it illustrates the influence of a minor change in the sample processing.

Efficiency of miRNA Primer Probes

We examined the efficiency of the miRNA primer probes in the arrays, and whether limits of detection by the probes affect

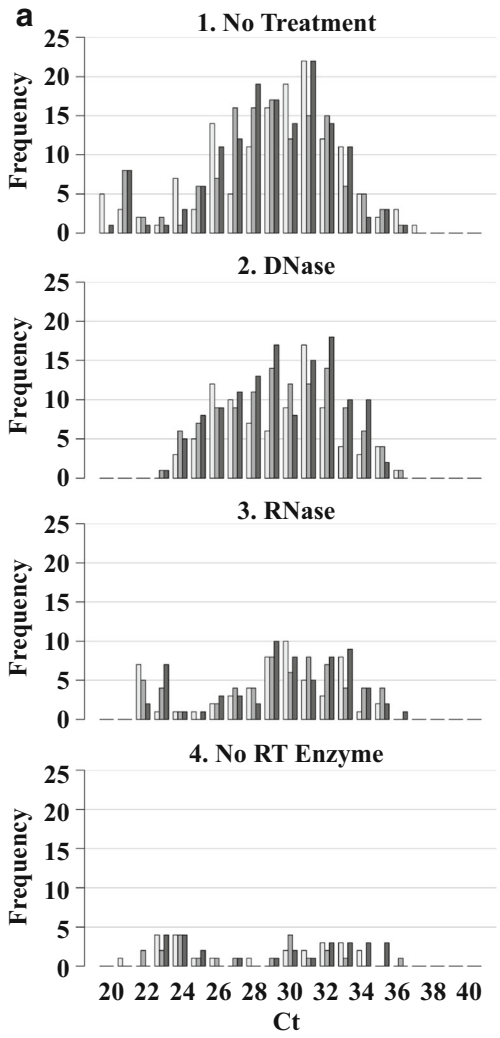

Fig. 1 **a** Test for non-specific amplifications in total RNA. The histograms show the outcomes from amplification of one identical RNA sample processed in one of four treatment groups: (1) no treatment (processed normally), (2) DNase treated, (3) RNase treated, or (4) no RT enzyme in the reverse transcription reaction. The colored bars (white, gray, black) represent one of the three RNA isolates in each treatment group. **b** Technical variations in each treatment group. Correlation matrix plot for the four treatment groups ($n = 3$ per group): (1) no treatment, (2) DNase treated, (3) RNase treated, or (4) no RT enzyme in the reverse transcription reaction. Axis labels are raw Ct values. Numerical blocks to the right of the axis are the correlation R^2 values; graphical blocks to the left of the axis are the miRNA correlation plots for each of the respective comparisons

qPCR outcomes. Each cycle of PCR leads to exponential growth in the number of DNA copies, thus the amount of DNA doubles with each Ct. However, amplification can be affected by factors including primer efficiency and the amount of input RNA. Thus, we analyzed outcomes using four input RNA quantities corresponding to a 0.1X, 0.3X, 1X, and 3X concentration; the 1.0X RNA represented the approximate amount of input used in our previous CSF studies [11]. We used regression analysis to establish the linear range for the miRNAs and determine the R^2 value, the p value, and the qPCR efficiency for each probe (Supplemental Table O–Q). Figure 3a shows an example of three outcomes from these studies. The left regression plot of miR-223 represents an optimal outcome for the miRNA amplification. The qPCR efficiency is 101.7%, the R^2 value is 0.99, and the Ct values change in direct proportion to the amount of input RNA. For reference, we include the effects of each treatment from Fig. 1 on Ct values. All three technical replicates of miR-223 amplified in the no treatment group (red circles), the DNase group (red diamonds), and the RNase group (red plus), but not in the no RT enzyme group (which would have been indicated by a red X). In contrast, the middle plot of miR-125a-5p suggests that the RNA inputs are at or near the detection threshold. Here, the qPCR efficiency is 121.7%, the R^2 value is 0.43, the 0.3X input amplifies but at varying Cts, and the 0.1X input does not amplify. For reference, we again include the Ct values measured in each treatment group in Fig. 1**.** Two of the three technical replicate for miR-125a-5p amplified in the no treatment group, one of three replicates amplified in the DNase group, and no replicates amplified in the RNase and no RT enzyme groups. The right plot of miR-645 represents a probe that is potentially saturated by the RNA at all input amounts. Here, the qPCR efficiency is negative 82.0%, the R^2 value is 0.62, and the Ct values change opposite to the RNA concentration. For reference, we again include the Ct values measured in each treatment group in Fig. 1, where three of the three technical replicates for miR-645 amplified in each of the no treatment, DNase, and RNase groups, but none of the no RT enzyme replicates amplified. Further, miR-645 amplified in the NTC sample at Ct 30.93, further evidence of non-specific amplification. Thus, this miRNA would be excluded from further analysis, in this study.

Effect of Individual TLDA Cards on qPCR Outcomes

We next examined whether variations in processing of the commercially available TLDA cards might affect outcome measures. We isolated total RNA from ten individual 0.5 mL aliquots of CSF, pooled the RNAs to create a uniform RNA starting sample, then made ten individual aliquots of the pooled RNA to perform the RT and amplification reactions. We calculated the mean Ct value, SD, Count, CoV, and lower and upper CI for each miRNA on the array (Supplemental Table R-W). All of the RT reactions were performed on the same date; however, half (five) of the cDNAs were pre-amplified on one date (group 1: 1.1–1.5), and half of the cDNAs were pre-amplified 8 days later (group 2: 2.1–2.5). Note that the RT and pre-amplifications were performed using the identical manufacturing lots of primers, enzymes, and master mix. A correlation plot showing that individual TLDA cards are not a source of variation within manufacturing lots is shown in Fig. 4a. We also used concordance scatter plots for group 1 vs group 2 to assess conformity of the results,

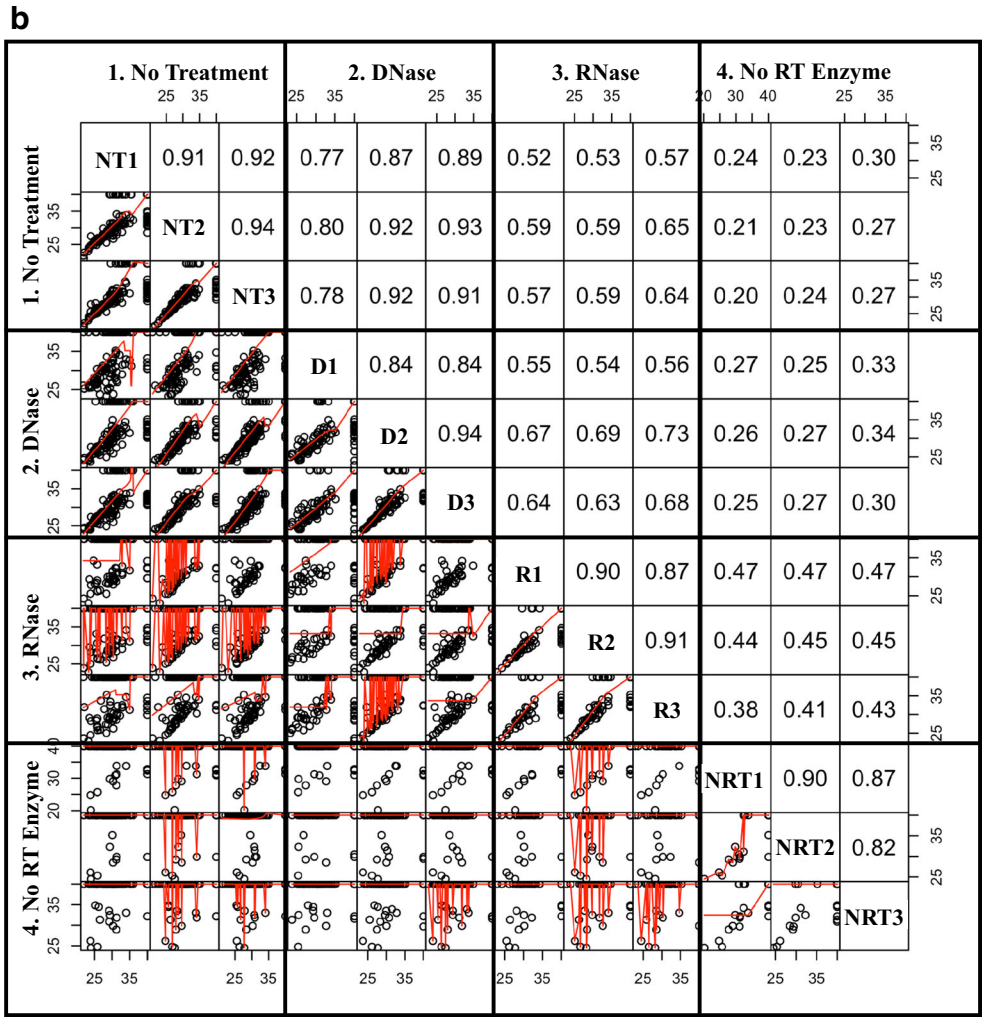

Fig. 1 (continued)

and in Fig. 4b each dot represents a single miRNA. The left plot represents the mean of the raw Ct values, while the right plot represents the mean of the card-adjusted Ct values. These results show that the TLDA cards within an identical manufacturing lot have little effect on miRNA expression outcomes.

Utilizing a Reference Standard to Correct for TLDA Card-to-Card Variations

Our findings indicate that day to day processing, including RNA isolations and preamplification, introduces variance in the data. To account for this batch effect in large-scale CSF studies, we include a uniform RefStd sample within each batch of RNA isolations performed on an experimental day, as a consistent, non-changing sample over the entirety of the study. Here, we present the results from the RefStd ($n = 18$ technical replicates, gray) among the experimental control samples ($n = 53$ experimental replicates, white), with vertical lines delineating individual batches (Fig. 5a). The boxplots contain trace curves that connect (i) the centers of the raw Cts for the RefStd replicates (black line connecting dark gray boxes) and (ii) the raw U6 Ct values for the RefStd replicates (dark gray line connecting dark gray dots). In experimental conditions void of technical error, the raw Cts and U6 values for the RefStd replicates would align. However, our data using the RefStd replicates show that the raw and U6 Cts can be up to about two or three Cts different between batches (Fig. 5a, top). To correct for this variance in experimental samples, we used the RefStd data to apply a batch centering to the results (Fig. 5a, middle), then followed up with a normalization based on U6 (Fig. 5a, bottom). The middle plot shows that the batch centering has the effect of aligning all the RefStd centers, which partially but imperfectly also lined up the RefStd U6 values. The bottom plot shows that the normalization has the additional effect of lining up all of the U6 values, placing the Ct values for every sample on the same scale. To assess whether the correction process had the desired effect of decreasing unwanted technical variance, we calculated coefficients of variation across the RefStd samples for each

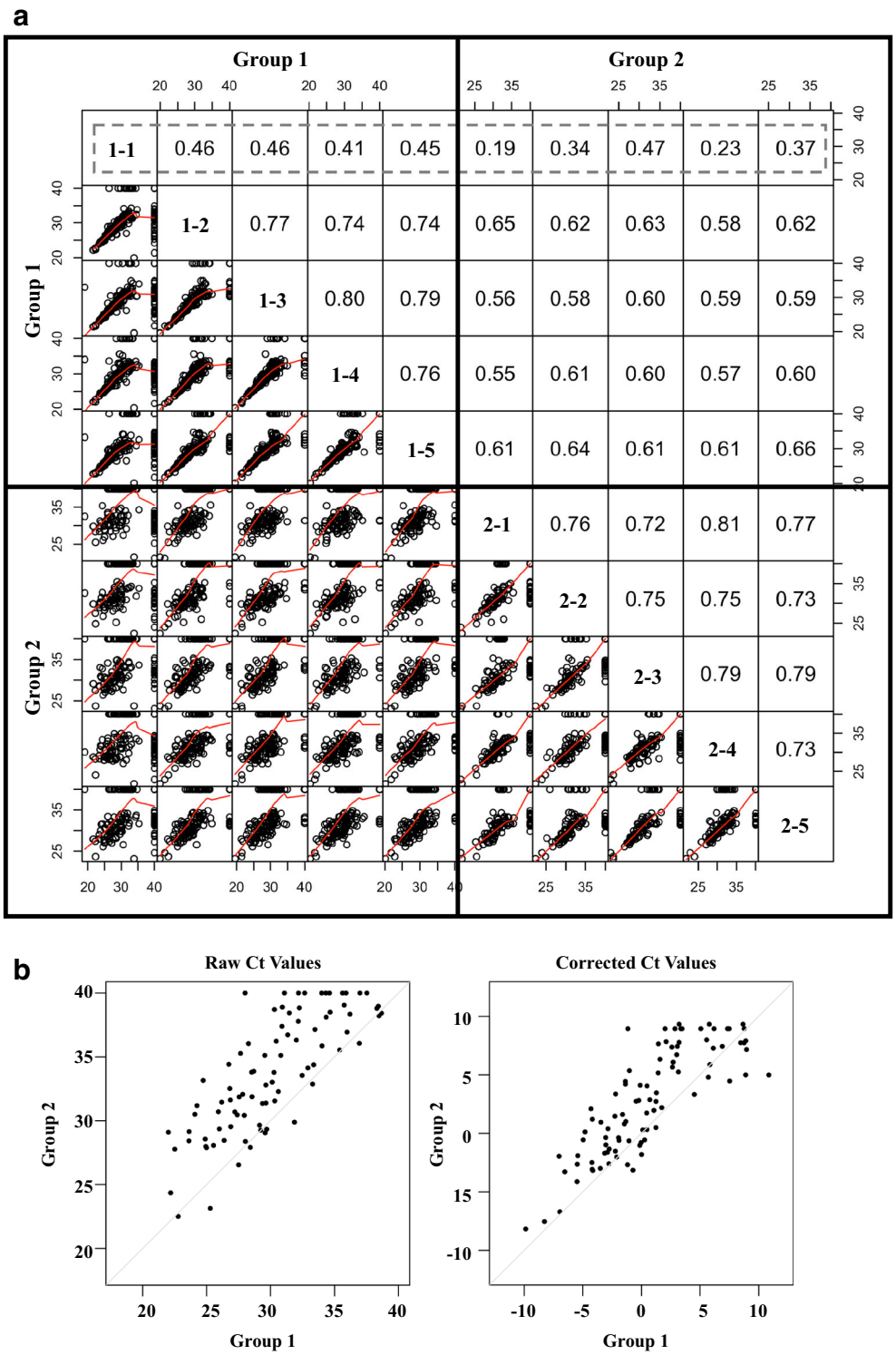

Fig. 2 **a** Technical variations in individual RNA isolations. Correlation matrix plot for two groups of five RNA samples isolated on two separate dates, designated as group 1 (1.1 to 1.5) and group 2 (2.1 to 2.5). Sample 1–1 indicated by a dashed line failed the PCR amplification. **b** Pearson correlation for group 1 vs group 2. The raw Ct plot (left) has a calculated 0.789 correlation between the two groups. The corrected Ct plot (right) has a calculated 0.821 correlation between the two groups

miRNA (Fig. 5b, sorted by raw Ct CoV). Raw CoV values ranged from 0 to 0.15 for nearly all miRNAs (black dots). Indicative of reduced technical variance, batch-adjusted values (gray dots) tended to fall below the corresponding raw values, and the batch-corrected and normalized values (hollow gray circles) almost always fell lower still. The mean

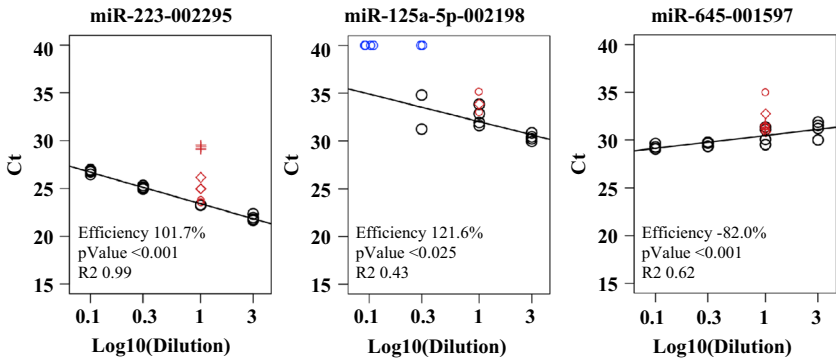

Fig. 3 Representative regression analysis of MiRNAs at four RNA inputs. The left graph is the regression plot for miR-223 that represents with a PCR efficiency of 101.7% and an R^2 value 0.99. The middle graph shows miR-125a-5p with a PCR efficiency of 121.6% and an R^2 value of 0.43; the right graph shows miR-645 with a negative PCR efficiency of − 82.0%. Symbols at the 1X concentration refer to amplification outcomes (Fig. 1). Good (black circles); censored (blue circles); amplified in no treatment group (red circles); amplified in DNase group (red diamond); amplified in RNase group (red +); amplified in no RT enzyme group (red X)

CoV for raw Cts was 0.064, but for batch-adjusted and batch-adjusted-plus-normalized Cts, it was 0.062 and 0.058, respectively. Although the correction process did not reduce the CoV in every single miRNA, for the vast majority of miRNAs technical error was mitigated. In the few instances where CoV was larger after the correction, it was still small in absolute terms. These results show that a uniform RefStd is useful for removing unwanted technical variations that can occur in large-scale studies that are performed over a period of weeks to months.

Discussion

Extracellular RNA expression studies using qPCR arrays are useful for the discovery and validation of biomarkers for human diseases [2]. However, there is a lack of reproducibility in outcomes between different vendors [15] making it difficult or impossible to compare outcomes from different laboratories. In addition, several factors within a laboratory can contribute to inconsistent results of qPCR studies. Here, we examined factors that could contribute to variations in outcomes in human CSF miRNA measures assessed using TLDA cards, and report five key findings from our studies.

First, there is evidence of nonspecific amplifications in the DNase, RNase, and no RT enzyme groups, corresponding to products amplified in the NTC assay (Supplemental Table E–H). The products removed by the DNase treatment and/or found in the NTC experiment may result from contamination introduced during the RNA isolation or qPCR process, based on the fact that even if primer-dimers or other nonspecific amplification products form, the TaqMan probes will not generate fluorescent signal [16]. DNA in the CSF samples may reflect that the samples were not centrifuged prior to storage and may have cellular contamination below the threshold of detection in the clinical assays. CSF is also known to contain circulating cell-free mitochondrial DNA (mtDNA). For example, the mtDNA is decreased in CSF from asymptomatic patients at risk of AD and symptomatic AD patients relative to controls [17], as well as in patients with early-stage Parkinson's disease relative to controls [18]. In contrast, significantly higher levels of mtDNA were found in the CSF of patients with relapsing-remitting multiple sclerosis [19]. Even if contaminating DNA is present in the sample, the stem-loop design of RT primers will deter primer annealing to genomic or mtDNA. We used RiboShredder for the RNase studies as it is advertised to degrade all RNAs, although the product is proprietary and the identity of the RNases in the product are unknown. Thus, the products remaining in the RNase group may reflect DNA in the samples, particularly as their amplification in the 22 Ct is in the range of those products lost in the DNase group. Based on our observation, we suggest that initial protocol refinement studies include a DNase and RNase treatment in order to identify products that amplify in these groups and to either remove these from further analysis or to flag and monitor their expression in diagnostic groups.

Second, we examined the effect of individual RNA isolations on qPCR outcomes. The results show that there is no significant effect on performance outcomes within individual RNA isolations on a given date. However, there are differences in performance between RNA isolations performed on separate dates, which may reflect differences in lab personnel or unanticipated variations in equipment performance. We used a card-wise normalization approach to increase the group-level concordance. In a large-scale study with hundreds of samples, this approach helps to minimize the "measurement noise" so that we have the sensitivity to measure differential outcomes more confidently. We previously reported the outcomes from evaluating RNA yield and purity using four RNA isolation kits in three independent laboratories, and found consistency in results between the different RNA isolation kits [20]. Wang et al. evaluated six RNA isolation methods, and developed a customized CSF-miRNA TLDA panel to evaluate CSF miRNA biomarkers with improved specificity,

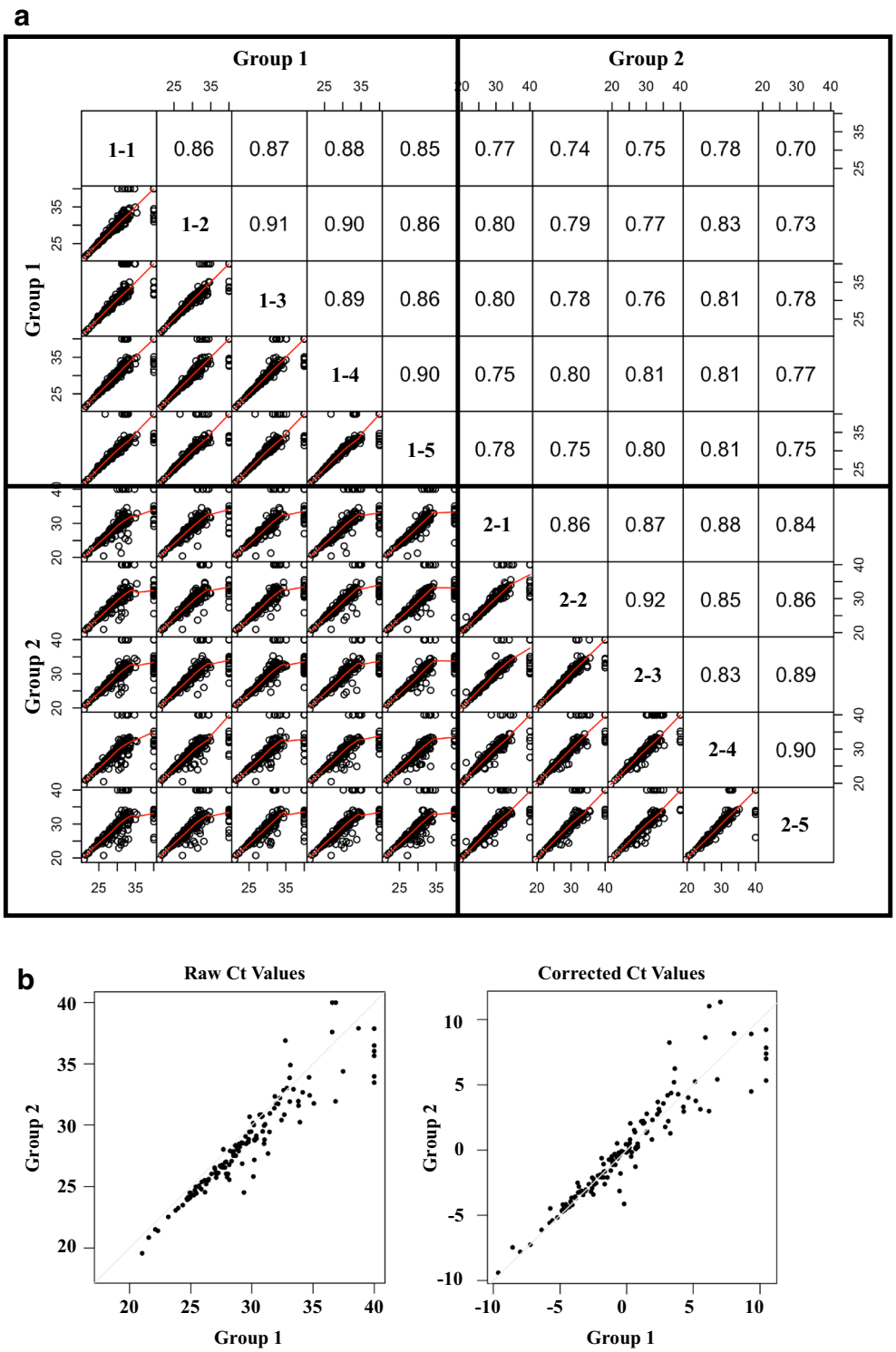

Fig. 4 **a** Technical variations in individual TLDA card processing. Correlation matrix plot for ten individual RT and pre-amplification reactions on pooled CSF RNA. Group 1 (G1) and group 2 (G2) were pre-amplified on two separate dates, with 8 days in between processing. **b** Concordance scatter plots for group 1 vs group. Each dot represents a single miRNA. The left plot represents the mean of the raw Ct values, while the right plot represents the mean of the card-adjusted Ct values

sensitivity, fast processing and data analysis, and cost effectiveness, that may be used for clinical diagnosis and disease stage monitoring [21].

Third, we determined the efficiency of the miRNA primer probes, in order to establish their limits of detection in the array and to determine if probe performance could lead to

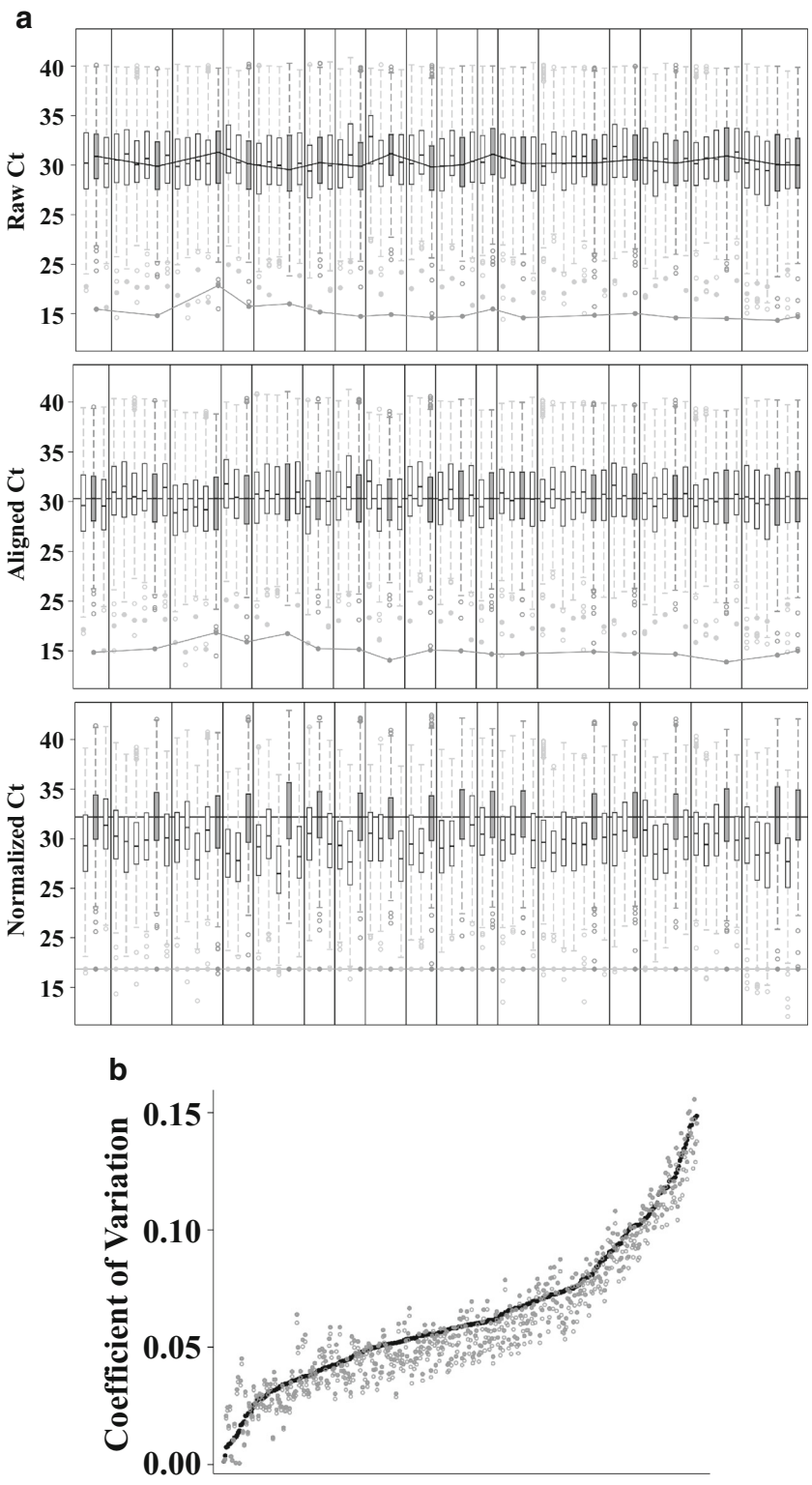

Fig. 5 **a** Effect of utilizing a reference standard to correct for TLDA card-to-card variations. **a** Box plot of raw Ct values (top). Vertical lines indicate the 17 batches; gray boxes are reference standard (technical) replicates ($n = 18$), white boxes represent experimental replicates ($n = 53$). Box plots after centering on within-batch reference standards (middle). Box plots after normalization (bottom). **b** Summary of coefficient of variation calculations across RefStd samples. Black dots are the pairwise CoV for the raw Ct, filled gray dots are the values for the RefStd centered data, and hollow dots are the values after both RefStd centering and U6 normalization. **b** Coefficients of variation across the RefStd samples for Each MiRNA. The graph shows raw CoV values for all miRNAs (black dots), batch-adjusted values (gray dots), and batch-corrected and normalized values (hollow gray circles) for all miRNAs. The mean CoV for raw Cts was 0.064, for batch-adjusted and batch-adjusted-plus-normalized Cts it was 0.062 and 0.058, respectively

inconsistent outcomes in the assays. RNAs that are abundantly represented in a sample may saturate PCR reagents, while RNAs expressed in low amounts may not be detected. Our results revealed those miRNAs that amplified across all RNA inputs and in accordance with increasing amounts of RNA. We also identified miRNAs where the RNA was too low for amplification at all input amounts. In contrast, there are miRNAs that appear to have saturated the assay even at the lowest input amount. Thus, performing a serial dilution with less input RNA, or performing single-tube reactions sign to

identify potential presence of inhibitor(s) in the assays, can be used to further assess the performance of the miRNA probs. These experiments are warranted particularly for those miRNA identified as potential miRNAs of interest in a large-scale study. Nevertheless, this aspect of a protocol refinement can provide context regarding the performance of the miRNA probes at each RNA input and thus the reliability of data from the specific miRNAs of interest in an experimental dataset. A recent study reported two workflows for determining a lower limit of quantitation for qPCR assays of miRNAs in exploratory studies: the first based on an error threshold calculated by a logistic model of the calibration curve data; the second based on a threshold set by the sample blank (NTC for qPCR) [22]. In our NTC data (Supplemental Table H), there is a correlation between amplification in the NTC assay and poor qPCR performance in the linear dilution series (Fig. 3). For example, miR-106B is in the NTC and it performed poorly in the dilution series ($R^2 = 0.27$, p value = 0.03, efficiency = 419%). Together, these data support that the miRNA probes or the qPCR amplification are not reliable, and these miRNAs would be flagged and observed closely in the experimental data or excluded from the final dataset in the analytic pipeline.

Fourth, we determined the effect of individual TLDA card performance on qPCR outcomes. Our results show that the TLDA cards do not contribute to variations in outcomes *within* identical lots of the array cards. However, we have previously found differences in outcomes *between* lots of the array cards [11]. Thus, maintaining consistency of reagent lots in qPCR studies is highly recommended for best results.

Fifth, we examined the effect of utilizing a uniform reference standard sample to correct for batch-to-batch variations in large-scale studies that require processing over weeks or months. We found that the cards can be aligned using the reference standard measures in order to center the sample distributions, after which the data sets can be normalized relative to endogenous controls or non-changing miRNAs. Normalization of qCPR data clearly effects the outcome and accuracy of the results. For miRNA studies, other small non-coding RNAs such as U6 are often used for normalization. However, use of the mean expression value of all expressed miRNAs in a given sample as a normalization factor is better at reducing technical variation and a more accurate appreciation of biological changes [23]. A subsequent study evaluated various methods of normalization to determine the optimal approach for quantifying miRNA expression from biofluids and tissue samples when using the TaqMan® Megaplex high-throughput qPCR platform with low RNA inputs [24]. The study compared seven normalization methods for analysis of variation of miRNA expression, and developed a novel variant of the common mean-centering normalization strategy; mean-centering restricted normalization [24].

One limitation of this study is that we did not include a spike-in control. However, Brunet-Vega et al. examined the robustness of miRNA purification and measurement by the addition of spike-ins and to evaluate the quality of the qPCR data [25]. They also evaluated adverse effects of inhibitors of reverse transcriptase and polymerase enzymes in biofluids on the quantification of circulating miRNAs [25]. A recent study provided four recommendations for miRNA profiling in plasma using TLDA cards: (i) implementation of a preamplification step in the TLDA protocol without diluting the final preamplification product; (ii) using a stepwise approach to exclude non-informative miRNA based on quality control parameters; (iii) argues against using U6 as normalization method for relative quantification; and (iv) using the geNorm algorithm as normalization method for relative quantification [26]. Based on these and our related study [20], we suggest that each new large-scale/long-term study incorporate a protocol refinement period. Further, we suggest implementing a stable control sample (for example, a large amount of pooled control "RefStd" CSF) in each RNA isolation batch such that corrections to the data can be made before normalization, which is particularly important when processing large numbers of experimental samples over a period of months. These efforts are intended to examine and identify factors in the assay or processing that may result in unintentional variations in outcome measures, and to ultimately increase confidence in the resulting data.

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