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A pilot study to troubleshoot quality control metrics when assessing circulating miRNA expression data reproducibility across study sites

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Abstract.
BACKGROUND: Given the growing interest in using microRNAs (miRNAs) as biomarkers of early disease, establishment of robust protocols and platforms for miRNA quantification in biological fluids is critical.
OBJECTIVE: The goal of this multi-center pilot study was to evaluate the reproducibility of NanoString nCounter™ technology when analyzing the abundance of miRNAs in plasma and cystic fluid from patients with pancreatic lesions.
METHODS: Using sample triplicates analyzed across three study sites, we assessed potential sources of variability (RNA isolation, sample processing/ligation, hybridization, and lot-to-lot variability) that may contribute to suboptimal reproducibility of miRNA abundance when using nCounter™, and evaluated expression of positive and negative controls, housekeeping genes, spike-in genes, and miRNAs.

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RESULTS: Positive controls showed a high correlation across samples from each site (median correlation coefficient, $r > 0.9$). Most negative control probes had expression levels below background. Housekeeping and spike-in genes each showed a similar distribution of expression and comparable pairwise correlation coefficients of replicate samples across sites. A total of 804 miRNAs showed a similar distribution of pairwise correlation coefficients between replicate samples ($p = 0.93$). After normalization and selecting miRNAs with expression levels above zero in 80% of samples, 55 miRNAs were identified; heatmap and principal component analysis revealed similar expression patterns and clustering in replicate samples.
CONCLUSIONS: Findings from this pilot investigation suggest the nCounter platform can yield reproducible results across study sites. This study underscores the importance of implementing quality control procedures when designing multi-center evaluations of miRNA abundance.

Keywords: MiRNA quantitation, biomarkers, reproducibility, biospecimens, pancreatic cancer

1. Introduction

Early detection and prevention strategies are desperately needed for pancreatic ductal adenocarcinoma (PDAC), a malignancy with a relative five-year survival rate of only 10% [1]. Molecular biomarkers from liquid biopsies, clinical parameters, and imaging tools hold promise for detecting preinvasive and early PDAC [2]. MicroRNAs (miRNAs) are excellent candidate molecular biomarkers of early cancer or preinvasive disease because of their tissue-specific expression patterns, remarkable protection from endogenous RNase activity, and their ability to regulate hundreds of tumor suppressors and oncogenes [3–5]. We and others have shown the potential clinical utility of miRNAs in the detection of PDAC and its cystic precursor lesions known as intraductal papillary mucinous neoplasms (IPMNs) [6–12]. Given the growing interest in the use of miRNAs as biomarkers for the detection of early-stage PDAC and high-grade IPMNs, the establishment of accurate, sensitive, specific, and reproducible platforms for circulating miRNA quantification is prudent, a goal in line with the National Cancer Institute's Early Detection Research Network [2]. However, studies are lacking in this important area.

The nCounter™ system (NanoString Technologies, Seattle, WA, USA) is an automated high-throughput platform designed to evaluate gene expression in clinical samples in a single multiplexed reaction that relies on hybridization of transcripts or miRNAs to probes conjugated to fluorescent barcodes [13,14]. In a single institutional study, our team [7] used nCounter™ technology to evaluate miRNA abundance in pre-operative plasma from patients with IPMNs as an alternative to microarray and polymerase chain reaction-based methods because of its potential to more accurately quantify low miRNA levels in blood without pre-amplification and its availability in research and certified clinical laboratories throughout the country. Using plasma and cystic fluid samples collected, processed, and banked using standard operating procedures (SOP) as part of a multicenter study [15], the objective of this pilot project was to troubleshoot potential methodological sources of variability that may contribute to suboptimal reproducibility of circulating miRNA abundance when using the nCounter™ system: RNA isolation, NanoString miRNA sample preparation/ligation, hybridization, and reporter probe lot variability.

2. Materials and methods

2.1. Study population

The Florida Pancreas Collaborative is a multi-center prospective cohort study established by a multidisciplinary team of investigators from three Florida academic cancer centers (Moffitt Cancer Center (MC), The University of Florida (UF), and Sylvester Cancer Center/University of Miami (UM)), with the goal of advancing prevention and early detection efforts for PDAC [15]. Between 9/3/2015 and 8/18/2018, each study site recruited males and females 18 to 99 years old presenting to the GI clinic, surgery, or endoscopy at Moffitt, UF, or UM with a clinical suspicion for (or diagnosis of) a pancreatic lesion, cyst, mass, or pancreatitis based on symptoms, imaging, or blood-work. Confirmation of the clinical diagnosis was made using histology, cytology, lab markers, and/or imaging. Informed consent was obtained under institutional review board-approved protocols at each institution and documented in the electronic medical record. This study was undertaken with the understanding and written consent of each subject, and the study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants were assigned a unique study identification (ID) number comprising the two-letter study site prefix followed by four numbers (ie. MC1234).

2.2. Biospecimen collection and processing

Blood was collected from consented participants via phlebotomy in a 10-mL EDTA tube and processed for plasma within 2 hours using standard procedures as described previously [7]. The tube of plasma was inverted three times, spun at 3,600 rpm for 8 minutes, aliquoted into 0.5-mL bar-coded cryovials, and banked at $-80°$C. Cystic fluid was collected at the time of an endoscopic ultrasound guided fine-needle aspiration (EUS-FNA) or at the time of surgical resection via aspiration, and then aliquoted and stored into 0.5 mL cryovials at $-80°$C.

2.3. Pilot design

This pilot study was designed to conduct the RNA isolation, processing/ligation, and hybridization steps in triplicate for a subset of 0.5 mL plasma or cyst fluid aliquots from our three study sites, with variation in the site performing certain steps (Table 1). Sample naming conventions were used and included the study ID followed by 'BB' for baseline blood/plasma, 'CF' for cystic fluid, and 'R' for replicate samples from each site, ending with a suffix of A (for MC), B for (UF), and C (for UM). We also evaluated high quality (QU) RNA reference samples using Ambion Universal Human Reference RNA derived from 10 human cell lines (ThermoFisher Scientific, Waltham MA) and pooled plasma donated by healthy controls (HC) recruited at the MC site. Since each NanoString nCounter cartridge allows 12 samples to be evaluated simultaneously, a total of 4 'BB' trios, 4 'CF' trios, 2 'QU' trios, and 2 'HC' trios were examined across the three study sites as shown in Table 1.

2.4. RNA isolation from plasma and cyst fluid and quality control

One 0.5 mL cryovial of plasma (and cyst fluid) was retrieved and thawed on ice from pilot study participants. The possibility of hemolysis (rupturing of erythrocytes) was assessed and recorded prior to RNA extraction since hemolysis can be a source of variation in studies of circulating miRNAs. Hemolysis assessment was performed based on recommended guidelines [1,16,17]. First, samples were visually inspected for a pink/red hue. Then, the NanoDrop spectrophotometer (NanoDrop Technologies, Waltham, MA) was used to measure and record hemoglobin absorbance at $\lambda = 414$, 541, and 576 nm (values exceeding 0.2 indicative of hemolysis). This hemolysis signature was also evaluated post-hoc for correlation with cellular miRNAs that are commonly elevated in the presence of hemolysis (miR-451, miR-16) [17].

Total RNA isolation was performed on 500 μL of plasma using the Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit, catalog #51000 (Slurry Format) from Norgen Biotek (Ontario, Canada). Modifications to the standard protocol included: 10 μL of β-mercaptoethanol was added to each 1 mL of Lysis Buffer (in a new 50 mL tube). Since the vendor protocol is specific for 1 mL input, volumes of slurry C2, Lysis Buffer, and Ethanol were adjusted to 0.2 mL, 0.8 mL, and 1.5 mL, respectively to accommodate 0.5 mL input. After thawing the sample on ice and evaluating for hemolysis, 800 μL of lysis buffer A (including beta-mercaptoethanol) and 200 uL of Slurry C2 were added and vortexed. After five minutes, spike-in-oligos (which had been thawed and kept on ice) were added. To control for variance in the starting material and the efficiency of RNA extraction, RNA spike-in miRNAs (synthetic control templates) were used. The spike-ins consisted of 1000 attomoles (5 μl of a 200 pM solution of each spike-in) of the synthetic RNA oligonucleotides (spike-in oligos) osa-miR-414, cel-mi-248, and ath-miR-159a (Operon, Inc, Huntsville, AL). RNA was eluted in 100 μL of water, concentrated to 20 μL using Amicon Ultra 0.5 mL MWCO 3 kDa centrifugal filters, catalog # UFC500324 (Sigma-Alrich, St. Louis, MO, USA), and 3 μL was used for each NanoString assay. Total RNA concentration and integrity were assessed using both NanoDrop and an Agilent Bioanalyzer RNA Pico Chip (Agilent, Santa Clara, CA, USA).

2.5. High-throughput measurement of miRNA abundance

The nCounter^{TM} Human v3 miRNA Expression Assay CodeSet (NanoString Technologies, Seattle, WA, USA) was used to quantify the abundance of 800 human miRNAs, 5 housekeeping genes (ACTB, B2M, GAPDH, RPL19, and RPLP0) and built-in controls: positive controls (spiked-in RNA at various concentrations to assess overall assay performance, $n = 6$) and negative controls (probes designed against synthetic sequences provided by the External RNA Controls Consortium (ERCC) for background calculation, $n = 8$). The input used was 3 μL of the extracted plasma and CF RNA and 100 ng of QU Reference RNA (as input) per manufacturer instructions available at https://www.nanostring.com/resources/tech-note-mirna

Table 1
Pilot study design for miRNA quality control work

		Moffitt Cartridge 1					UF Cartridge 1					UM Cartridge 1				
				Task to be performed and by whom:					Task to be performed and by whom:					Task to be performed and by whom:		
Description	Diagnosis	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization
Baseline plasma replicate sample from Moffitt participant MC0008	IPMN															
– Moffitt to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		1	MC0008_BB_R1A	MC	MC	MC	1	MC0008_BB_R1B	MC	MC	UF	1	MC0008_BB_R1C	MC	MC	UM
High quality replicate RNA sample 1	N/A															
– Moffitt A27 perform ligation on one aliquot and then send an 8 uL aliquot to the other 2 sites for the hybridization step		2	QU001_R1A	n/a	MC	MC	2	QU001_R1B	n/a	MC	UF	2	QU001_R1C	n/a	MC	UM
Healthy control plasma replicate sample 1	Control															
– Moffitt to perform RNA isolation on one plasma sample and then send a 5 uL aliquot to the other 2 sites for ligation and hybridiz		3	HC001_BB_R1A	MC	MC	MC	3	HC001_BB_R1B	MC	UF	UF	3	HC001_BB_R1C	MC	UM	UM

Table 1, continued

		Moffitt Cartridge 1					UF Cartridge 1					UM Cartridge 1				
				Task to be performed and by whom:					Task to be performed and by whom:					Task to be performed and by whom:		
Description	Diagnosis	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization
Cyst fluid replicate sample from UF participant UF011	Pseudocyst															
– UF to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		4	UF011_CF_R1A	UF	UF	MC	4	UF011_CF_R1B	UF	UF	UF	4	UF011_CF_R1C	UF	UF	UM
Baseline plasma replicate sample from Moffitt participant MC0033	IPMN															
– Moffitt to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		5	MC0033_BB_R1A	MC	MC	MC	5	MC0033_BB_R1B	MC	MC	UF	5	MC0033_BB_R1C	MC	MC	UM
Cyst fluid replicate sample from UF participant UF012	Pseudocyst															
– UF to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		6	UF012_CF_R1A	UF	UF	MC	6	UF012_CF_R1B	UF	UF	UF	6	UF012_CF_R1C	UF	UF	UM

Table 1, continued

		Moffitt Cartridge 1					UF Cartridge 1					UM Cartridge 1				
				Task to be performed and by whom:					Task to be performed and by whom:					Task to be performed and by whom:		
Description	Diagnosis	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization
Cyst fluid replicate sample from UM participant UM054	Pseudocyst															
– UM to Isolate RNA and perform ligation on one cyst fluid sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		7	UM054_CF_R1A	UM	UM	MC	7	UM054_CF_R1B	UM	UM	UF	7	UM054_CF_R1C	UM	UM	UM
Baseline plasma replicate sample from UM participant UM064	Pseudocyst															
– UM to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step		8	UM064_BB_R1A	UM	UM	MC	8	UM064_BB_R1B	UM	UM	UF	8	UM064_BB_R1C	UM	UM	UM
High quality replicate RNA sample 2	N/A															
– Each site performs ligation and hybridization on the aliquot received		9	QU002_R1A	n/a	MC	MC	9	QU002_R1B	n/a	UF	UF	9	QU002_R1C	n/a	UM	UM

Table 1, continued

Description	Diagnosis	Moffitt Cartridge 1					UF Cartridge 1					UM Cartridge 1				
				Task to be performed and by whom:					Task to be performed and by whom:					Task to be performed and by whom:		
		Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization	Sample #	FPC_ID	RNA isolation	Sample preparation	Hybridization
Healthy control plasma replicate sample 2 – Each site performs RNA isolation, ligation, and hybridization on the aliquot received.	Control	10	HC002_BB_R1A	MC	MC	MC	10	HC002_BB_R1B	UF	UF	UF	10	HC002_BB_R1C	UM	UM	UM
Baseline plasma replicate sample from UM participant UM024 – UM to Isolate RNA and perform ligation on one plasma sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step	IPMN	11	UM024_BB_R1A	UM	UM	MC	11	UM024_BB_R1B	UM	UM	UF	11	UM024_BB_R1C	UM	UM	UM
Cyst fluid replicate sample from UF participant UF013 – UF to isolate RNA and perform ligation on one cyst fluid sample and then send an 8 uL aliquot to the other 2 sites for the hybridization step	Pseudocyst	12	UF013_CF_R1A	UF	UF	MC	12	UF013_CF_R1B	UF	UF	UF	12	UF013_CF_R1C	UF	UF	UM

-analysis-in-plasma-and-serum/. Preparation involved multiplexed annealing, a ligation reaction of synthetic DNA sequences (miRTags) to mature miRNAs through sequence-specific bridges, an enzymatic purification to remove excess miRTags, and a final dilution where 40 μL of RNAase free water was added to each sample.

The samples were then stored at $-20°$C until hybridization, where 5 μL of the tagged miRNA probes were hybridized to a color-coded capture and reporter probe pair. After post-hybridization processing on the NanoString PrepStation, following transfer of the immobilized nCounter cartridge to the nCounter Digital Analyzer, reporter probes were counted for each miRNA at 555 fields of view. Of note, reporter probes in the cartridge delivered to one site (MC) had a different lot number than cartridges delivered by NanoString to the other two sites (UF and UM).

2.6. Data processing and quality control

Raw counts of each target were log2-transformed. For each sample, background-corrected measures of miRNA expression were calculated by subtracting the negative control mean plus two standard deviations (SD) from the raw miRNA counts. Human messenger RNA (mRNA) housekeeping genes included in the CodeSet (ACTB, B2M, GAPDH, RPL19 and RPLP0) were used to evaluate possible cellular sample contamination, as these genes should be very low or non-detectable in cell-free samples. Data for each sample were normalized using the geometric mean of 3 spike-in oligos. Since it is important to consider assay performance when using different lots of reagents, lot-to-lot variability was accounted for by generating a calibration/scaling factor based on a set of common biological samples that expressed the majority of targets of interest above background using manufacturer-suggested procedures available in NanoString data analysis guidelines.

2.7. Data analysis

Statistical methods used in this investigation included (a) the Kruskal-Wallis test for group differences among study sites and specimen types, (b) principal component analysis (PCA) for data reduction of endogenous miRNA expression, and (c) correlation analysis with Spearman correlation for housekeeping and spike-in genes and Pearson correlation for endogenous genes to evaluate the correlation of miRNA expression between sites. Graphical visualization included (a) boxplots for distribution of gene and miRNA expression and correlation coefficients among study sites and specimen types, (b) heatmap for patterns of gene and miRNA expression and correlation coefficients, (c) scatterplots for correlation of gene and miRNA expression and PCA analysis, and (d) barplot for PCA analysis. All statistical analyses were performed using the R 3.6.0 software (https://www.R-project.org).

3. Results

Of the 12 pre-treatment samples evaluated across the study sites, 8 were from participants with the following pancreatic conditions: IPMNs ($n = 3$) and pseudocysts occurring in the background of pancreatitis ($n = 5$). The remaining four samples evaluated across sites derive from healthy controls without pancreatic conditions ($n = 2$) and high-quality RNA samples (QU) from a manufacturer ($n = 2$) (Table 1). RNA quality control metrics and electropherograms are featured in Supplementary Table 1 and Supplementary Fig. 1, respectively. The six positive controls built in to the nCounter platform were highly correlated across samples from each of the study sites (Supplementary Fig. 2). The median correlations with the empirical 95% confidence interval (95%CI) were 0.96 (95%CI: 0.95–0.98) in MC, 0.93 (95%CI: 0.90–0.96) in UM, and 0.97 (95%CI: 0.95–1.0) in UF. The 8 built-in negative control probes had expression below background noise (< 32) in most samples across sites (Supplementary Fig. 3) except two outliers in the NEG_C negative control with expression values above the background in 5 of 12 samples at MC and in 1 of 12 samples for the NEG_H negative control at UM.

The five housekeeping genes showed a similar distribution of expression among the three study sites ($p > 0.05$; Supplementary Fig. 4). In general, expression was highest in the high-quality RNA samples (QU) followed by the cyst fluid (CF) samples and the plasma samples (BB). The distribution of pairwise correlation coefficients of replicated samples were comparable ($p = 0.41$) among the three sites. The median correlation coefficient was 0.8 in MC vs UF, 0.9 in MC vs UM, and 0.85 in UM vs UF (Supplementary Table 2). Correlation coefficients were the highest in the QU samples followed by the BB, and the smallest in the CF samples.

Of five spike-in miRNA probes that can be detected by the nCounter panel, we used three for normalization at the manufacturer's suggestion: osa-miR-414, cel-mi-248, and ath-miR-159a. The spike-in miRNA probes

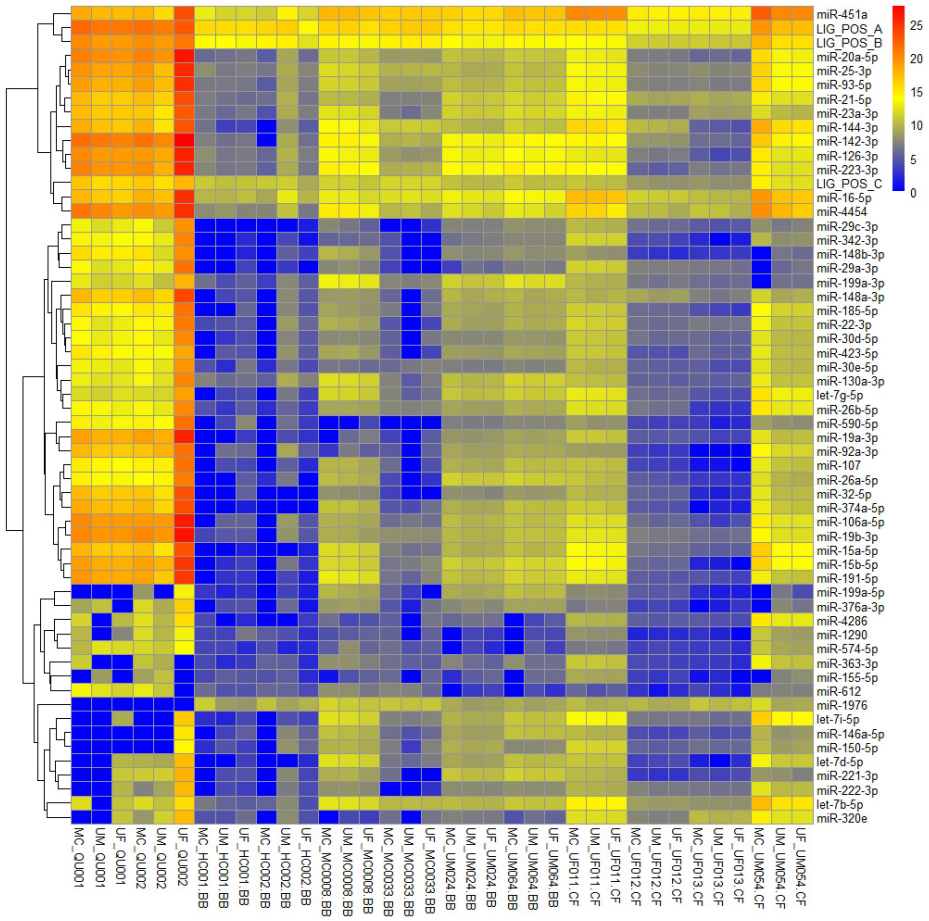

Fig. 1. Heatmap for the 55 miRNA targets and the 3 ligation controls that had expression above zero across $>$ 80% of samples.

had a comparable distribution of expression among the three study sites ($p > 0.05$; Supplementary Fig. 5). (The other two probes, cel-mi-254 and osa-miR-442, were not spiked in and therefore did not have detectable levels.) Expression levels were the highest in the CF samples, then in the BB, and the smallest in the QU samples for these three spike-in genes, as expected since spike-ins were not added to QU RNA samples. Distribution of pairwise correlation coefficients of replicate samples was comparable ($p = 0.40$) among the three sites. The median correlation coefficient was 1.0 in MC vs UF, 0.9 in MC vs UM, and 0.9 in UM vs UF. The correlation coefficients were the highest in the cyst fluid samples, then in the plasma samples, and the smallest in the high-quality RNA samples (Supplementary Table 3).

For the $\sim$ 800 endogenous miRNA targets, distribution of pairwise correlation coefficients between replicate samples were comparable ($p = 0.93$) among the three sites (Supplementary Fig. 6). The median correlation coefficient was 0.9 in each paired site comparison (i.e., MC vs UF, MC vs UM, and UM vs UF). After normalization, a total of 55 miRNA targets (plus 3 ligation controls) had expression levels above zero in 80% of samples across study sites. A heatmap for these targets shows that most trios had similar expression patterns across sites (Fig. 1). Furthermore, samples tended to cluster by disease/diagnosis type (IPMN vs. Pseudocyst vs. Healthy Control) and sample type (BB vs CF) (Fig. 2). Importantly, some of the identified miRNAs such as miR-126, miR-574 and miR-1290 have been highlighted as biomarkers of high-grade IPMNs or early pancreatic cancer by our team and others (Fig. 1) [6–12]. Principal component analysis (PCA) for the 55 miRNAs revealed that the first principal component (PC1) accounts for 69.5% of the variability in the data; PCA also showcased clustering of sample trios (Supplementary Fig. 7). Distribution of pairwise correlation coefficients between replicate samples were also comparable ($p = 0.68$) among the three sites (Fig. 3). The median correlation coefficient was 0.9 in MC vs

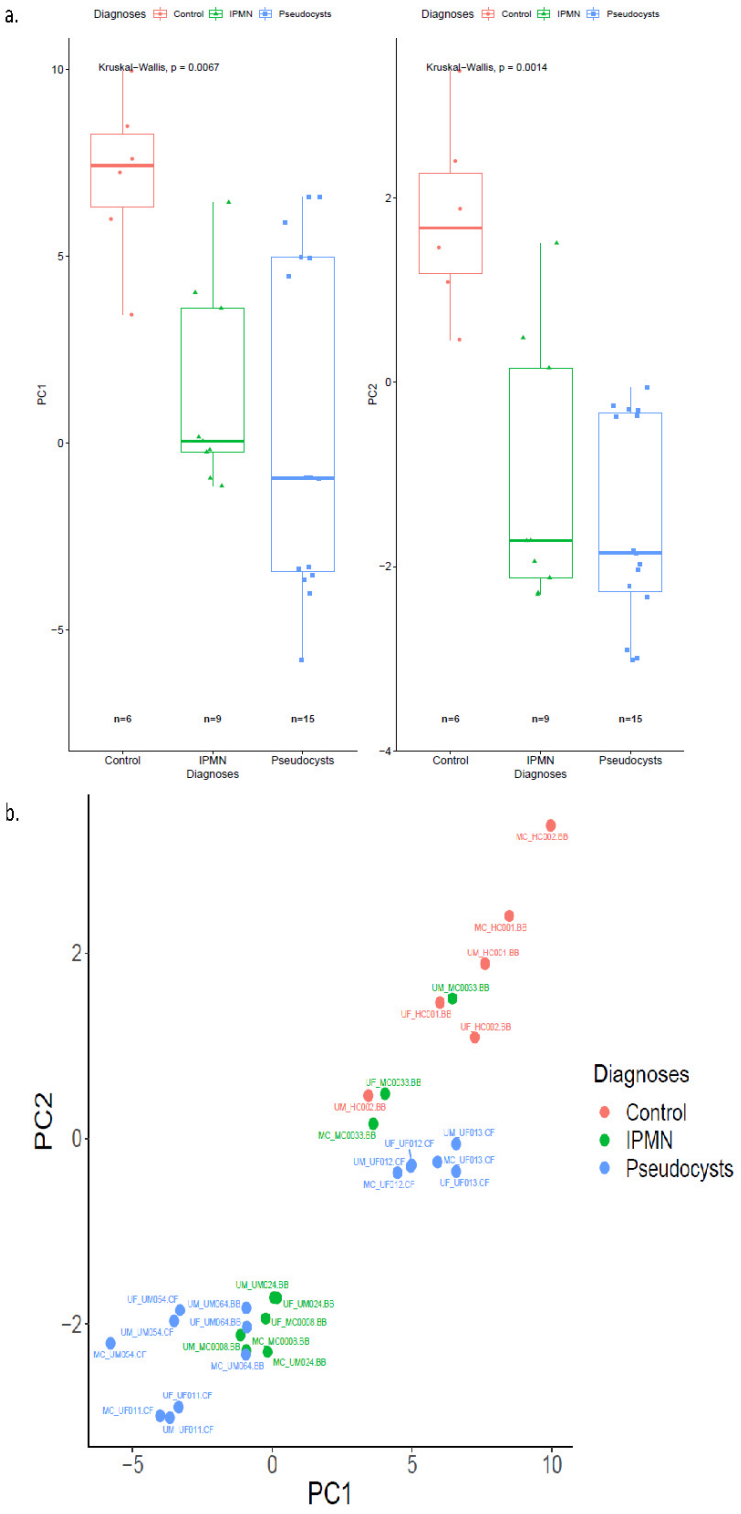

Fig. 2. Endogenous expression of 55 miRNAs and 3 ligation controls in study samples characterized by principal components for each diagnosis type represented by a) box plots and b) scatterplots.

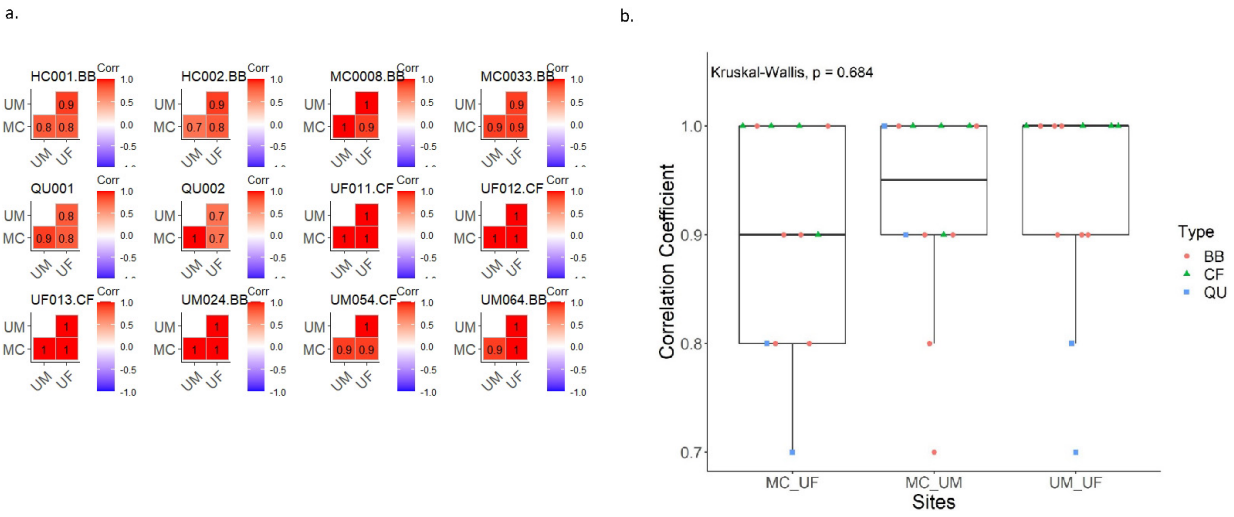

Fig. 3. Pearson correlations of the 55 miRNA targets expressed in study samples across sites. a) Correlation coefficient matrix bar plot for each sample, b) Correlation coefficient boxplot.

UF, 0.95 in MC vs UM, and 1.0 in UM vs UF. The miRNA expression signals and correlation coefficients were higher in the CF samples compared to the BB and the QU samples, possibly due to higher RNA yield. (Supplementary Fig. 8).

4. Discussion

Liquid biopsies based on miRNA profiles have potential to aid in early detection efforts for pancreatic cancer. However, it is necessary to account for methodological sources of variability that may confound results and contribute to suboptimal reproducibility of miRNA expression in human samples as part of multi-center studies. On a subset of plasma and cyst fluid samples, this multi-center pilot study applied standardized methodology for RNA isolation, processing, and hybridization using nCounter technology (NanoString Technologies, Inc) across study sites. Overall, we report strong correlations in quality control metrics such as positive and negative control probes, housekeeping genes, and spike-in probes. Additionally, endogenous miRNAs above background were well correlated across trios of samples evaluated across study sites.

Despite promising data, we observed some variations in quality control metrics across the three sites that might be explained by inconsistencies in sample preparation, equipment, or normal site-to-site variability. To explore these concerns in more detail, a field application scientist from NanoString (EAP) evaluated the raw data and staff from the Moffitt Molecular Genomics Core (SJY, TM) re-visited the spike-in dilution calculations and sent follow-up questions to each site to elucidate reasons for the differences. Reasons for the variance in the spike-ins and positive controls included having different technicians perform the ligation step and site to site differences in thermocycler brand and calibration. Additionally, the spike-in dilutions were about 40% lower than when the assay was performed in a single-institution study years prior [7], explaining not only the lower dilution counts but also the variation in the final volume after concentrating samples. It was later learned that the high-quality control RNA was not diluted at UF prior to subsequent steps. This pilot study highlights the need for careful attention to following a standardized protocol.

Despite these nuances, our proof of concept results suggest that miRNA quantitation can be reliably reproduced across study sites with the nCounter platform, opening the door for robust miRNA-based signature development in cell-free samples such as plasma and ascites fluid. Next steps include incorporating the lessons learned and continuing to evaluate miRNA expression profiles from plasma and cyst fluid samples from newly-diagnosed cases with IPMNs and other types of pancreatic lesions across study sites. Such work holds great promise for advancing early detection efforts for pancreatic cancer.

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Supervision: JBP, SLW, SJY

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-210255.

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