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miRNA Expression in Ovarian Cancer in Fresh Frozen, Formalin-fixed Paraffin-embedded and Plasma Samples

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Abstract. *Background/Aim: MicroRNAs (miRNAs) are small noncoding RNAs involved in gene expression regulation and have been investigated as potential biomarkers for various diseases, including ovarian cancer (OC). However, lack of standardized protocols regarding e.g., RNA isolation, cDNA synthesis, spike-in controls for experimental steps, and data normalization, impacts cross validation of results across research groups and hinders implementation of miRNAs as clinical biomarkers. Materials and Methods: RNA was isolated from matching fresh-frozen tissue (FF), formalin-fixed paraffin embedded (FFPE) tissue, and plasma samples from twenty women diagnosed with OC using three commercial kits: miRNeasy Tissue/Cells, miRNeasy FFPE, and miRNeasy Serum/Plasma (Qiagen, Copenhagen, Denmark). RNA isolation, cDNA synthesis, and PCR performance were tested using miRCURY LNA miRNA Quality Control PCR (QC) Panels (Qiagen). Finally, miRNA stability was assessed using five algorithms: BestKeeper, Normfinder, GeNorm, comparative delta-Ct and comprehensive ranking provided by a web-based RefFinder tool. Results: RNA from FF, FFPE and plasma was extracted using commercially available kits and the differences in yield and purity were examined. We developed a simple method for identifying and potentially excluding samples based on their crossing point values from RT-qPCR data, which could improve existing manufacture guidelines. Moreover, we discussed how assessment of miRNA stability differs between algorithms, possibly leading to inconsistent results. Conclusion: We present guidelines for RNA isolation, cDNA synthesis, and data normalization for successful miRNA expression profiling using RT-qPCR in corresponding biological OC specimens. We recommend QC panels in combination with spike-in controls and interplate controls to monitor process efficiencies.*

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Key Words: miRNA, epithelial ovarian cancer, normalization, spike-in controls, RT-qPCR.

Ovarian cancer (OC) is the most lethal type of gynecological cancer causing 4.2% (age-standardized rates) of cancer related deaths worldwide (1). About 90-95% of OCs are epithelial OCs (EOCs), which consist of four main subtypes: serous, endometrioid, ovarian clear cell carcinoma, and mucinous EOC comprising 75%, 10%, 10%, and 3% of EOCs, respectively (2). OC is subdivided into 4 stages by the International Federation of Gynecology and Obstetrics (FIGO). Due to its covert growth, OC frequently goes undetected for an extensive initial period (3) and the majority of EOCs are diagnosed in late stages (FIGO, stage III or IV), which significantly impacts the overall survival (OS) of the patients (4, 5). As OC is a complex disease with specific treatments for different subtypes, a plethora of tests and analysis are needed to determine *e.g.*, cancer type, stage, grade, genetic background, and histology (2, 6).

MicroRNAs (miRNAs) are small noncoding RNAs that function in regulation of gene expression. Although the mechanism of regulation through the RNA Induced Silencing Complex (RISC) is well known, the cross reactivity and sheer number of complementary sequences of miRNA and mRNA make it difficult to assign specific roles to individual miRNAs (7). Moreover, given that each miRNA has a wide range of targets, disturbance in their expression can cause a variety of adverse effects (8, 9).

In recent years, the possibility of using miRNAs as biomarkers for different diseases and illnesses including OC, has therefore been explored (10, 11). Despite numerous studies having found several miRNAs related to various

diseases, including OC, implementation of miRNA biomarkers in clinical diagnostics has been hindered, in part due to the lack of well-established standardized protocols (12-14).

Expression results obtained from microarray or other high throughput methods are generally validated by RT-qPCR as a gold standard for gene expression (15, 16), as a result of well-established reference genes (17, 18). It has however, proved challenging to identify universally stable miRNAs to use as references in expression studies (19).

Although its unstable expression in cancers has been reported (20-23), U6 (*RNU6*), is commonly used as endogenous control when studying miRNA in OC (24-26). Many different algorithms and software exist to evaluate the stability of references, however, the results are not always in concordance, potentially leading to inconsistencies in normalization and predispose to discrepancies in conclusions between research groups (27).

The aim of our study was to suggest a complete RT-qPCR workflow that covers all steps from miRNA isolation to data normalization based on a platform from Qiagen, which showed optimal results in a previous comparative study (28).

Materials and Methods

Study design. A total of 20 matched samples of fresh frozen (FF), formalin-fixed paraffin embedded (FFPE) tumor tissue samples and blood plasma were collected from patients diagnosed with OC, FIGO stage III and IV, enrolled in two studies: Pelvic Mass and GOVEC (Table I). Patients have provided written informed consent. The study has been approved by the Danish National Committee for Research Ethics, Capital Region (KF01-227/03 and KF01-143/04) and followed the guidelines from the Declaration of Helsinki.

miRNA extraction of FF tissue samples. miRNA extraction from FF tissue samples was performed using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, Copenhagen, Denmark, cat. no. 217684) according to the manufacture's protocol with small adjustments regarding homogenization. For each sample, a piece of approximately 3-4 mm^3 was dissected from the FF tissue specimen and transferred to a 1.5 ml reaction tube with 60 µl lysis buffer containing 1% β-mercaptoethanol. The sample was then homogenized with a disposable polypropylene pestle, before adding the remaining 200 µl lysis buffer containing 1 µl RNA isolation spike-in control mix consisting of a 2 fmol/µl UniSp2, 0.02 fmol/µl UniSp4 and 2×10^{-4} fmol/µl UniSp5. All following steps were performed according to the manufacturer's protocol. The RNA was eluted in 15 µl RNase free water.

Two empty control extractions were also performed and produced identically to, and together with regular FF tissue extractions, except no tissue was added to the lysis buffer. These controls received the same treatment and spike-in RNAs as the tissue samples.

miRNA extraction of FFPE samples. Total RNA was extracted from two FFPE tissue slices (each 5 µm thick) using miRNeasy FFPE kit (Qiagen). Briefly, each paraffin block was sliced into two sections of 5 µm thickness. Paraffin was removed using 160 µl deparaffinization solution (Qiagen), followed by incubation for 3 min at 56˚C, before adding Proteinase K Digestion Buffer (Buffer PKD) and 1 µl RNA isolation spike-in control mix (Qiagen). Further steps were performed according to the manufacture's protocol. Finally, dried RNA, including miRNA, was dissolved in 20 µl RNase-free water.

Table I. *Clinicopathological characteristics of 20 epithelial ovarian cancer patients*.

Status	
Histology	
High grade serous adenocarcinoma	11
Low-grade serous adenocarcinoma	2
Carcinosarcoma, homolog	2
Endometrial adenocarcinoma	1
Clear cell adenocarcinoma	4
FIGO stage	
III	13 (65.0%)
IV	7 (35.0%)

FIGO: International Federation of Gynecology and Obstetrics.

miRNA extraction of plasma samples. For plasma samples, miRNA extraction was performed using the miRNeasy Serum/Plasma Kit (Qiagen). As a starting point, 200 µl of serum was lysed by adding 1 ml QIAzol lysis reagent, followed by addition of 1 µl RNA isolation spike-in control mix. Afterwards, the samples were purified according to the manufacturer's recommendations. The RNA was eluted in 14 µl RNase free water.

RNA quantification. The RNA concentration of FF and FFPE samples was measured using the Qubit RNA HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). In addition, concentration, purity, and contamination were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). All quantifications were performed using 1:10 dilutions.

cDNA synthesis. Reverse transcription of RNA was performed using the miRCURY LNA RT Kit (Qiagen, cat. no. 339340). For FF and FFPE samples, the RNA concentration for individual samples was adjusted to 5 ng/µl and 2 µl was added to 10 µl reactions including 0.5 µl cDNA synthesis spike-in control mix containing UniSp6 and cel-miR-39-3p. Instead of adjusting the no tissue controls to 5 ng/µl these were serial diluted 1:10, 1:100, 1:200, and 1:400. Two µl of each dilution was then used for cDNA synthesis. A fixed volume of 1.1 µl input RNA eluate from each sample was added to each 10 µl reactions also containing the cDNA synthesis spike-in control mix.

RT-qPCR. RT-qPCR reactions were performed using miRCURY LNA SYBR Green PCR Kit (Qiagen), miRCURY LNA miRNA QC PCR Panels, in a 384-well plate format (Qiagen) and a LightCycler 480 (Roche, Hvidovre, Denmark). The QC PCR panels contain twelve locked nucleic acid (LNA) PCR assays for detection of 1) the RNA isolation spike-in controls added at the beginning of each isolation procedure (UniSp2, UniSp4, and UniSp5), 2) the cDNA synthesis spike-in controls (UniSp6 and cel-miR-39-3p), 3) UniSp3, the interplate calibrator (UniSp3_IPC), to identify between runs variations, and 4) six miRNAs: hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-191-5p, hsa-miR-23a-3p, hsa-miR-30c-5p, and hsa-miR-45a. These miRNAs are widely expressed across different tissue

Table II. *List of miRNAs included in the miRCURY LNA miRNA QC PCR Panel. miRbase entry names are given. Target sequences were confirmed up to date as of version 22.1.*

miRname (human)	microRNA target sequence	Corresponding LNA™ microRNA PCR primer set (Qiagen Prod No)	Human, miRbase v20	Assay type
UniSp2		YP00203950		Spike
UniSp4		YP00203953		Spike
UniSp5		YP00203955		Spike
UniSp6		YP00203954		Spike
cel-miR-39-3p		YP00203952		Spike
UniSp3_IPC		YP02119288		IPC
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	YP00204772	hsa-miR-23a-3p	GOI
hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC	YP00204783	hsa-miR-30c-5p	GOI
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	YP00204063	hsa-miR-103a-3p	GOI
hsa-miR-124-3p	UAAGGCACGCGGUGAAUGCC	YP00206026	hsa-miR-124-3p	GOI
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	YP00204306	hsa-miR-191-5p	GOI
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU	YP02119305	hsa-miR-451a	GOI

types and biofluids (29). Hsa-miR-451a and hsa-miR-23a-3p are used as hemolysis markers for plasma samples (30). A list of miRNAs analyzed can be found in Table II. RT-qPCR reactions were prepared according to the manufacture's protocol. Briefly, a large pool containing the 2x miRCURY SYBR Green Master Mix and RNase free water was prepared, and the pool was then aliquoted into 32 individual microcentrifuge tubes where cDNA was added at a ratio of 1:200. The mixture was then homogenized thoroughly and briefly centrifuged before distributing 10 μl in to 12 individual PCR wells. The no tissue controls were treated identically to the samples; however, each dilution was run in duplicates on two different plates.

The PCR plates were sealed, centrifuged for 1 min at 1,500 × *g*, and subjected to real-time PCR amplification according to the protocol, including 2 min heat activation at 95˚C, 45 amplification cycles at 95˚C for 10 s and 56˚C for 60 s ending with a melt curve analysis. For each run the baseline and the Crossing points (Cps) of the amplification curves were calculated using the LightCycler®480 software version 1.5 (Roche) and absolute quantification analysis/2nd derivative maximum method with high confidence setting.

Data analysis. All data analyses were performed using R Statistical Software (version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria)(31) and R-studio IDE (version 1.4.1717; RStudio, Boston, MA, USA). Concentration comparisons were calculated using a paired two-sided Wilcoxon test with a significant level of 0.05. Cp values recorded at the 40th cycle were removed from the analysis. The Cps of each plate were then adjusted based on UniSp3_IPC. The minimum and maximum Cp values from two technical replicates for each of two no tissue controls (Table III) were recorded to define the accepted Cp range for FF samples and exclude potential outliers. After adjustments and validation, a Cp cutoff value of 35 was chosen.

The stability of the miRNAs investigated was assessed utilizing the online expression stability tool RefFinder (32) found at (33). The tool combines the analysis of several well-known algorithms; BestKeeper (34), Normfinder (35), GeNorm (36) and the comparative delta-Ct method (37). In addition, RefFinder assigns a weight to the ranking of the four statistical algorithms and calculates a geometric mean to determine an overall ranking of the analyzed genes.

Table III. *Assessment of acceptable range of Cp values. UniSp2 and UniSp4 Cp values from two serial dilutions of no tissue control samples (CT1 and CT2) produced alongside normal isolation. RT-qPCR was performed in two replicates for each control, denoted as a and b.*

	Dilution	UniSp2	UniSp4
CT1a	10	24.65	31.52
	100	29.27	35.51
	200	29.97	35.65
	400	30.61	36.44
CT1b	10	24.36	33.45
	100	27.84	
	200	29.07	
	400	29.88	
CT2a	10	26.11	30.95
	100	30.23	36.49
	200	31.34	35.01
	400	31.54	
CT2b	10	25.31	32.76
	100	29.16	35.61
	200	30.53	
	400	31.28	36.73
Mean		28.82	34.56
SD		2.431	2.052
CoV		8.44%	5.94%
Max		31.54	36.73
Min		24.36	30.95
Range		7.18	5.78

Results

Patients. Clinical and pathologic information on the patients is summarized in Table I. Thirteen women were categorized as FIGO stage III and seven as FIGO stage IV. Outcome of the patients was recorded on the 2nd of November 2021.

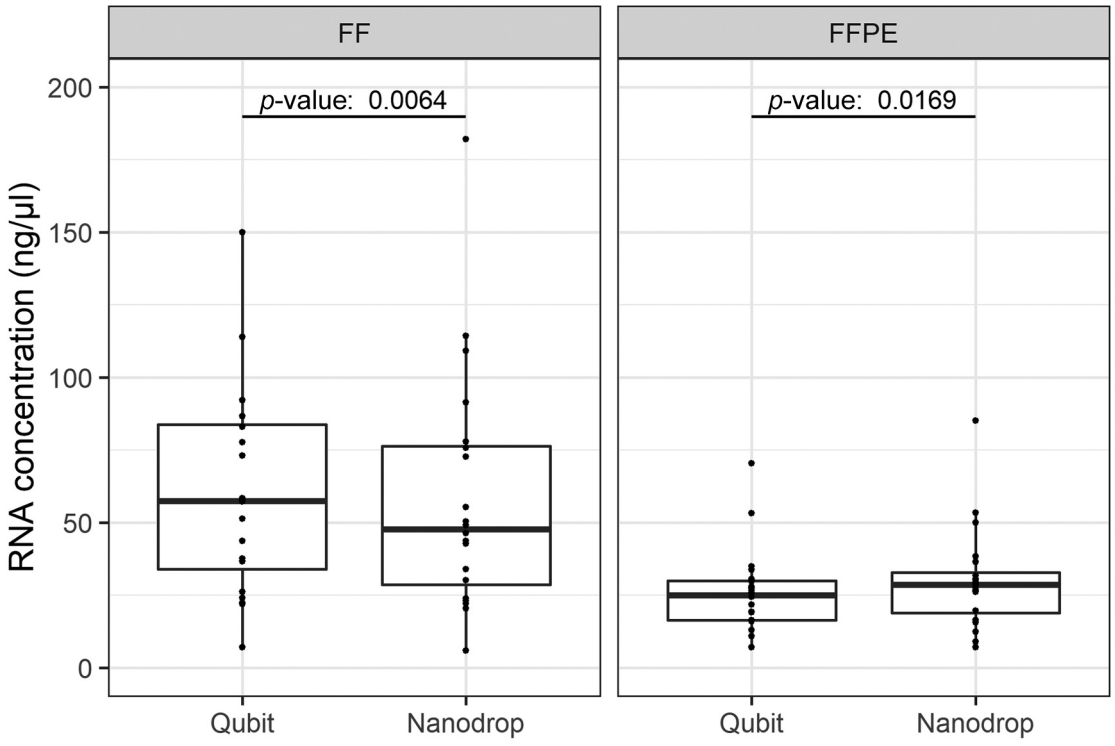

Figure 1. *Comparison of concentration measurements of 1:10 dilutions using Nanodrop and Qubit. Fresh frozen (FF) (left), formalin fixed, and paraffin embedded (FFPE) (right).*

RNA isolation and quantification. RNA from FF, FFPE and plasma was extracted using 3 commercially available kits: miRNeasy Tissue/Cells, miRNeasy FFPE and miRNeasy Serum/Plasma from Qiagen.

Since the concentration of miRNAs in the plasma is very low, Qubit and Nanodrop measurements were carried out only for FF and FFPE samples (Figure 1). To accommodate the analytical range of the Qubit Fluorometer, the RNA eluates were diluted 1:10 before quantification. The concentration of RNA from the 20 FF tissues were measured (mean: 63.57 ng/µl, range=7.1-150 ng//µl). To adjust the concentration of the FF samples to 5 ng/µl, dilutions ranged from approximately 1:14 to 1:300.

The Nanodrop measured concentrations between 5.99 ng/µl and 182.1 ng/µl (mean 58.55 ng/µl). The concentrations of the 20 FFPE samples ranged between 7.04 ng/µl and 70.4 ng/µl with a mean of 26.24 ng/µl when using Qubit and between 7.12 ng/µl and 85.2 ng/µl with a mean of 30.17 ng/µl when using Nanodrop (Figure 1). Significant differences in Qubit and Nanodrop concentration measurements were found (FF: p=0.0064 and FFPE: p=0.0169) (Figure 1). RNA purity and sample contamination were assessed using Nanodrop. For FF samples the A260/A280 ratio was between 1.85 and 2.01 with a mean of 1.94. The A260/A230 ratio of the same samples was between 0.348 and 2.08 with a mean of 1.24. For FFPE samples the A260/A280 ratio was between 1.76 and 2.08 with a mean of 1.89. The A260/A230 ratio of the same samples was between 0.80 and 2.32 with a mean of 1.77. A single sample (FFPE_09) turned out to be a low outlier, removal of this sample resulted in a range from 1.42 to 2.32 with a mean of 1.82 (Figure 2).

RT-qPCR. The miRCURY LNA miRNA QC PCR Panel was utilized to evaluate the quality of isolated RNA. After interplate calibration with UniSp3_IPC, the isolation spike-in controls (UniSp2, UniSp4, and UniSp5) and the cDNA synthesis controls (UniSp6 and cel-miR-39-3p) were then used to evaluate the efficiency of the process (Figure 3). UniSp2 and UniSp4, added during the lysis step of isolation, were observed in all samples, except for sample FF_20. UniSp5 was detected in all plasma samples but only in a few FF and FFPE samples (FF_01, FF_04, FF_12, FFPE_02, FFPE_05, FFPE_10, FFPE_11, FFPE_15, FFPE_16, FFPE_17 and FFPE_19).

Evaluation of RNA isolation spike-in controls. After interplate calibration, the minimum Cp value recorded for UniSp2 in FF samples was 24.37 cycles and the maximum was 28.83 cycles. Since the range obtained from the no tissue control was 24.36-31.54 cycles, these were all within the acceptable range for the given dilutions (Table III). For UniSp4, the obtained range for the FF samples was 31.53-

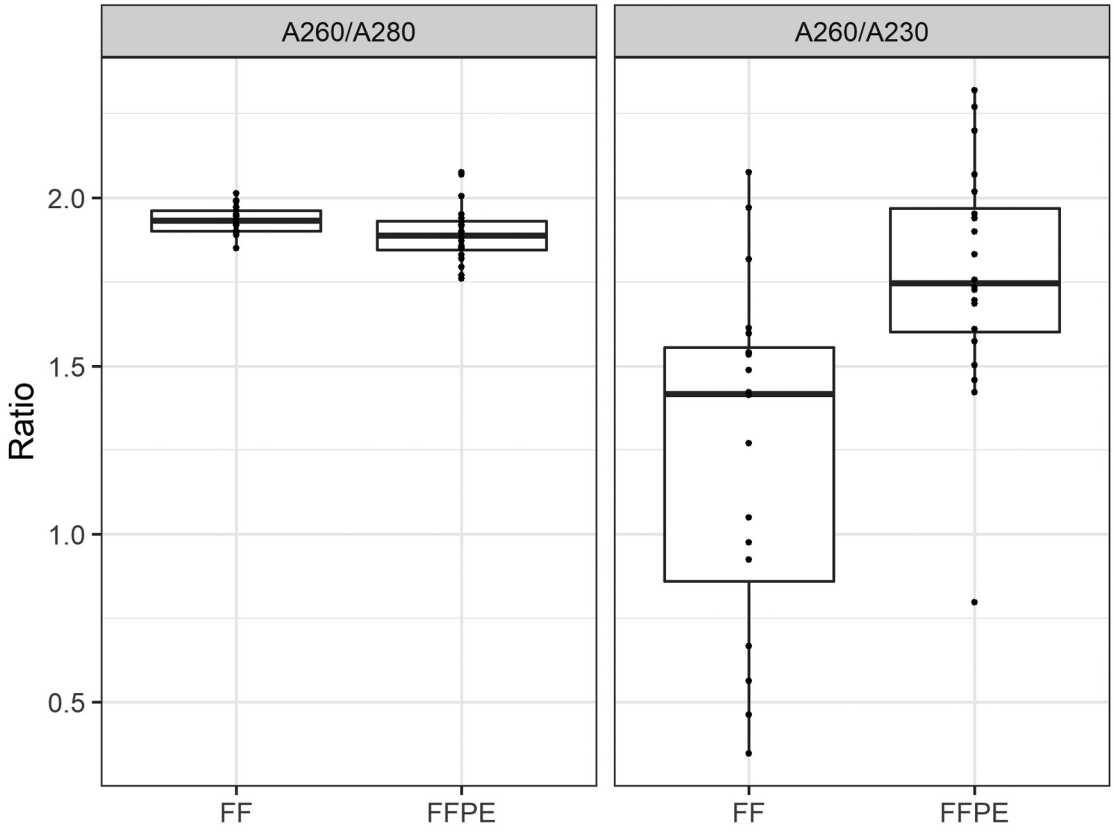

Figure 2. *Nanodrop assessment of purity (left) and contamination (right) for Fresh frozen (FF), formalin fixed, and paraffin embedded (FFPE) samples.*

35.17 cycles, which was within the minimum – maximum range for no tissue controls (30.95-36.73 cycles).

After adjusting for UniSp3_IPC, all UniSp5 measurement values in FF and FFPE samples were found to be above the threshold of 35 cycles and were therefore excluded from further analysis.

In plasma, 9 samples showed Cp values for UniSp5 below 35 after UniSp3_IPC adjustment. For FF samples, the mean difference between UniSp2 and UniSp4 was 7.42 Cp. This is 12.4% higher than the expected 6.6 Cp. For FFPE, this difference was 7.28, or 10.3% higher than expected. In plasma, the difference was 7.18 or 8.8% higher than expected.

The cDNA synthesis spike-ins (UniSp6 and cel-miR-39-3p) and the UniSp3_IPC were detected at Cp<35 in all samples. All samples presented call rates above 80%.

Hemolysis assessment of plasma samples. To test for hemolysis in the blood plasma samples, we subtracted the Cp values of hsa-miR-23a-3p from the Cp values of hsa-miR-451a (Figure 4). The results showed a range from −4.49 to 2.87 with a mean ΔCp of −0.81; no ΔCp above 7 was observed, so no hemolysis was detected.

Stability of controls and endogenous miRNAs. To assess the stability of cDNA synthesis spike-in controls, UniSp3_IPC and endogenous miRNAs in the different groups of samples, we used five algorithms: BestKeeper (34), Normfinder (35), GeNorm (36), the comparative delta-Ct method (37), and comprehensive ranking from RefFinder (32). The stability rankings were performed separately for each sample type (FF, FFPE, plasma) (Figure 5 and Table IV) and in groups ("FF+FFPE" or "FF+FFPE+plasma") (Table IV).

The stability of the tested miRNAs varied between tissue types and algorithms. For each individual group of FF, FFPE or plasma, different endogenous miRNAs were ranked as most stable. Delta-Ct, Normfinder, and the comprehensive ranking reported hsa-miR-30c-5p as the most stable endogenous miRNA in FF, whereas BestKeeper and GeNorm reported this miRNA to be the second most stable. BestKeeper and GeNorm ranked hsa-miR-103a-3p as the most stable endogenous miRNA, but it was reported as 3rd and 4th most stable according to delta-Ct and Normfinder, respectively.

All algorithms ranked hsa-miR-191-5p and hsa-miR-30c-5p as the two most stable endogenous miRNA in FFPE. The algorithms, however, did not agree on the specific ranking

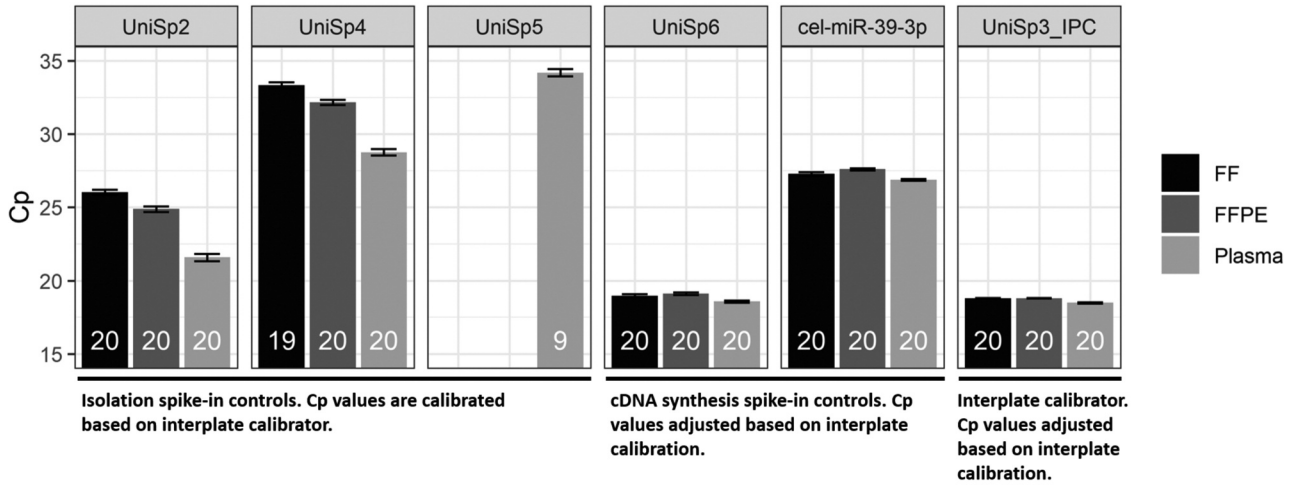

Figure 3. *Spike-in control analysis. Calibrated RT-qPCR Cp values below the 35-cycle threshold of RNA spike-in controls for fresh frozen (FF), formalin fixed, paraffin embedded (FFPE) and plasma samples. Number of samples, in which Cp was determined, is presented on each bar. Note that bars are truncated at Cp=15 to increase visibility of error bars.*

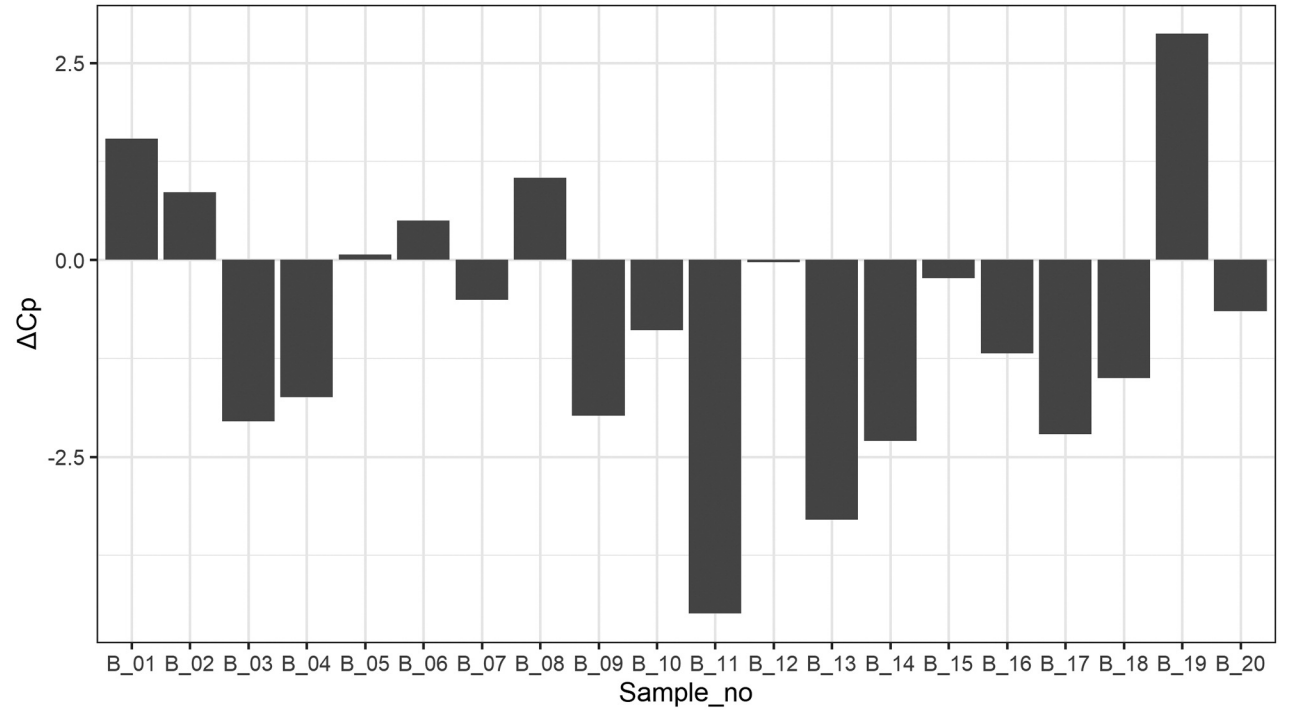

Figure 4. *Hemolysis analysis. Difference in Cp values of hsa-miR-23a-3p and hsa-miR-451a are given as ΔCp. No hemolysis is seen; values above 7 indicates hemolysis in the sample.*

when considering all measured controls and miRNAs. In this way, hsa-miR-30c-5p is considered as the 5th most stable by most of the algorithms except for Normfinder that reported this as the 2nd most stable of all miRNAs (Figure 5 and Table IV).

When looking only at plasma, hsa-miR-191-5p again was found to be the most stable endogenous miRNA, except for BestKeeper which found it to be the 5th most stable of all investigated miRNA. The second most stable of all miRNAs was hsa-miR-23a-3p based on four algorithms: Delta-Ct, Normfinder, GeNorm, and the comprehensive ranking. According to the BestKeeper ranking, hsa-miR-23a-3p was found to be the 6th most stable of all. When combining FF

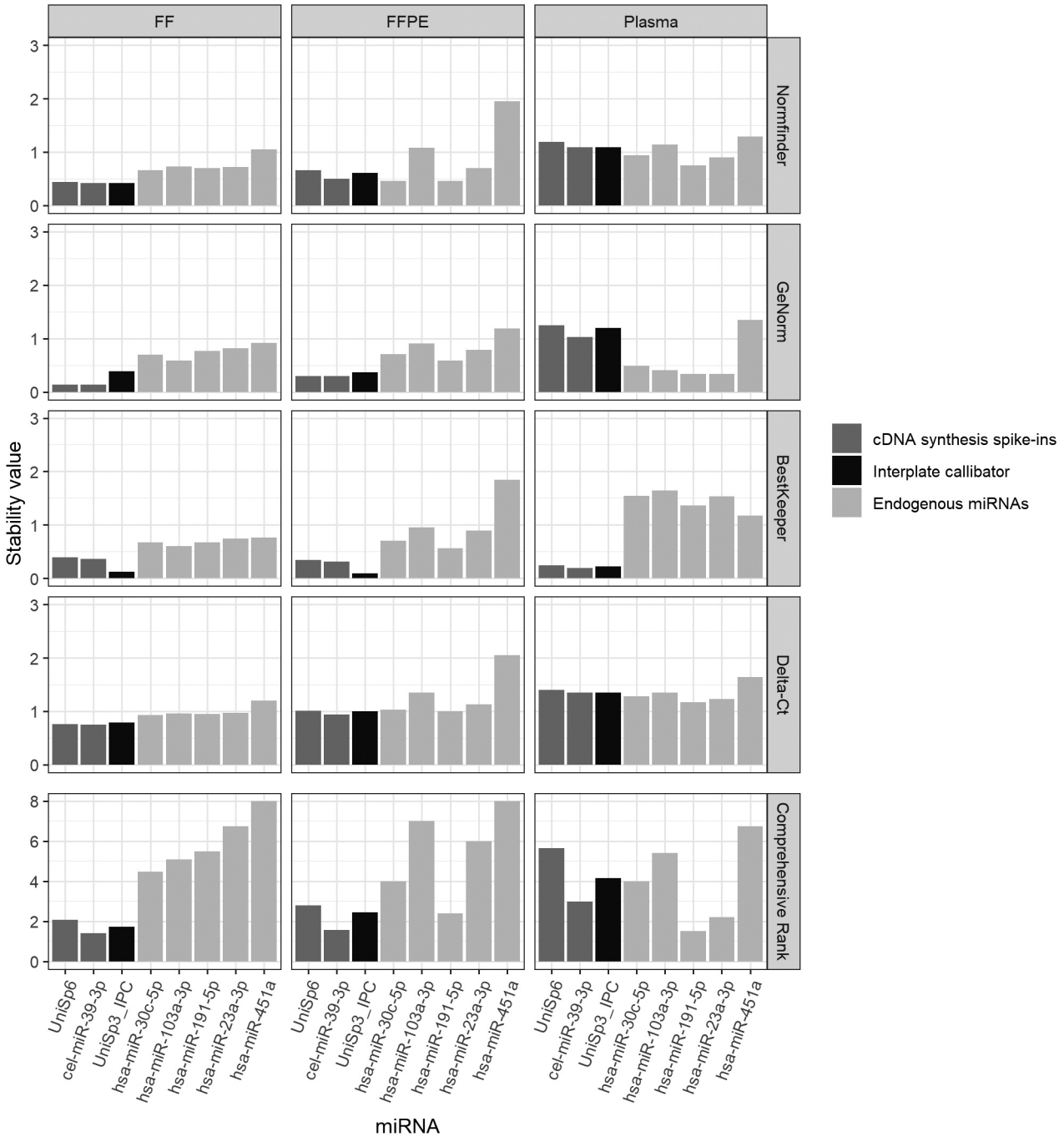

Figure 5. *Overview of normalization algorithms presented by RefFinder on FF, formalin fixed, paraffin embedded (FFPE) and plasma samples. Low stability values indicate more stable miRNAs. The comprehensive rank is a geometric mean of the four other algorithm stabilities. miRNAs colored dark grey are cDNA synthesis controls. The miRNA colored in black is the interplate calibrator. Light grey colors indicate endogenous miRNAs.*

and FFPE data, hsa-miR-30c-5p and hsa-miR-191-5p were shown to be the most stable, which agreed with the stability rankings for individual groups. In FF+FFPE+plasma group, the most stable endogenous miRNAs followed what was observed for plasma alone in that hsa-miR-191-5p and hsa-miR-23a-3p were reported to be the most stable. For all groupings, hsa-miR-451a was considered of low stability according to all algorithms.

Table IV. *Ranking of individual miRNAs of different groupings by different algorithms. Low values indicate more stable miRNAs. The two lowest scores for each algorithm in the spike-in control and the endogenous miRNAs groups are shown in bold.*

	FF					FFPE					Plasma					FF_FFPE					FF_FFPE_Plasma				
	DC	BK	NF	GN	CR	DC	BK	NF	GN	CR	DC	BK	NF	GN	CR	DC	BK	NF	GN	CR	DC	BK	NF	GN	CR
Spike-in controls																									
UniSp6	**2**	3	3	**1**	3	4	3	5	**1**	4	7	3	7	7	7	**4**	**2**	**5**	**1**	**3**	**2**	**2**	**4**	**1**	**2**
cel-miR-39-3p	**1**	**2**	**2**	**1**	**1**	**1**	**2**	**3**	**1**	**1**	**4**	**1**	**4**	**5**	**3**	**1**	3	**3**	**1**	**1**	**1**	3	**2**	**1**	**1**
UniSp3_IPC	3	**1**	**1**	3	**2**	**3**	**1**	**4**	3	**3**	**5**	**2**	**5**	**6**	**5**	6	**1**	6	3	4	4	**1**	5	3	4
Endogenous																									
hsa-miR-30c-5p	**4**	**5**	**4**	**5**	**4**	**5**	**5**	**2**	**5**	**5**	3	7	3	4	4	**2**	**5**	**1**	**4**	**2**	7	7	7	7	7
hsa-miR-103a-3p	6	**4**	7	**4**	**5**	7	7	7	7	7	6	8	6	3	6	7	6	7	7	7	6	**5**	6	6	6
hsa-miR-191-5p	**5**	6	**5**	6	6	**2**	**4**	**1**	**4**	**2**	**1**	**5**	**1**	**1**	**1**	**3**	**4**	**2**	**5**	**5**	**3**	**4**	**1**	**4**	**3**
hsa-miR-23a-3p	7	7	6	7	7	6	6	6	6	6	**2**	6	**2**	**1**	**2**	5	7	4	6	6	**5**	6	**3**	**5**	**5**
hsa-miR-451a	8	8	8	8	8	8	8	8	8	8	8	**4**	8	8	8	8	8	8	8	8	8	8	8	8	8

DC: Delta-Ct method; BK: BestKeeper; NF: Normfinder; GN: GeNorm; CR: RefFinders comprehensive ranking calculating a geometric mean based on the rankings from the other four algorithms.

Discussion

For a long time, RT-qPCR has been the gold standard for evaluating expression of various molecules in a countless number of specimens (38-40). The successful investigation of miRNA expression requires a robust and generalized consensus of a long range of factors concerning every part of the workflow from sample acquisition and handling through cohort selection, miRNA extraction methods, sample storage conditions to experimental procedures and data analysis, including methods for identification of endogenous miRNA controls (41). The literature indicates a general discord on what constitutes a robust RT-qPCR analysis, especially regarding miRNA expression studies (42). Therefore, we aimed on providing guidelines for a complete workflow from RNA isolation, cDNA synthesis and data normalization to successful miRNA RT-qPCR quantification for OC specimens: FF, FFPE and plasma.

When comparing RNA concentrations measured using Qubit and Nanodrop, we found that the Qubit tended to measure slightly higher concentration on FF samples than Nanodrop. Interestingly, we found the opposite tendency for FFPE samples. Though the Qubit was less prone to providing erroneous results due to the use of an RNA specific binding dye, the Nanodrop provides additional information in the form of the A260/A280 and the A260/A230 ratios, which serve as a measure of RNA purity and an assessment of potential contamination. Moreover, Nanodrop measurements are easier, faster, cheaper to perform and potentially use less material. We therefore suggest using the Qubit for the most accurate concentration measurement prior to standardization of RNA input to reverse transcription and, if needed, the use of the Nanodrop for estimating possible contamination of the samples. We found that the A260/A280 for both FF and FFPE tissues were commonly measured just below the favorable ratio of 2 indicating that the isolates were generally free of DNA and protein contaminations (Figure 2, left). In contrast, especially for FF tissues the A260/A230 varied from 0.35 to 2.08 denoting large variation in contaminations from organic compounds such as guanidine salts and phenol, which could interfere with downstream applications like cDNA synthesis and RT-qPCR (43). The FFPE tissues recurrently provided better and more reliable A260/A230 ratios (between 0.80 and 2.32) with only a single sample below 1.42 indicating that the washing and cleaning steps are more efficient when isolating from FFPE tissues than from FF.

The concentrations of FF samples measured using Qubit varied between 7.1 ng/μl and 150 ng/μl. The concentrations of FFPE samples varied between 7.04 ng/μl and 70.4 ng/μl. High variation in yields of FF samples could be attributed to variations in the sample size of the FF tissue (Figure 1). Although the amount of fixed tissue in FFPE blocks varies, the amount of specimen used for RNA isolation is much more controlled since slicing thickness is set mechanically. One solution could be to weigh the input material and use a fixed amount of tissue. However, this is impractical in a routine setting, the duration from cutting to lysis would increase and relatively large variation would still have to be tolerated due to differences in *e.g.*, cellularity. Therefore, no steps in the procedure would be eliminated and the gain from weighing the material would be minimal. This solution would also not be practical for FFPE samples since slices of the FFPE block are fragile and would not account for the extra material in the form of paraffin.

Since the concentrations of isolated RNA varied widely, and the input RNA amount for cDNA synthesis had to be adjusted to 10 ng in total, the cDNA samples were prepared with different RNA dilution ratios, leading to relatively high variation in observed UniSp2 and UniSp4 levels.

The 2-3 Cp difference exclusion range used by others in the literature and the manufacturer's guidelines (29, 44) did not account for variation in dilution. We therefore prepared serial dilutions of the RNA from the two no-tissue controls, providing us with an expected Cp range for the isolation spike-in controls and provided a method for excluding samples with Cp values for UniSP2 outside this range. To our knowledge this approach has not yet been utilized for the miRCURY LNA miRNA QC PCR Panels. In our study, we found that the Cp values obtained from the UniSp4 did not follow the expected pattern. In one case the Cp value for the 1:100 dilution was higher than that for the 1:200 dilution (Table III). Also, 5 out 16 no tissue control samples were missing values for UniSp4. These findings indicate that for highly diluted samples the UniSp4 may not be an optimal indicator of sample quality. Instead, this indicates that Cp data becomes unreliable when the Cp values reach approximately 30-35 cycles (45). A Cp value of 35 corresponds to about 10 template copies causing the technical variation to increase too much for reliable quantification (46) and when considering UniSp5, which is 100 times more diluted then UniSp4, this problem is only elevated. Only very few samples showed any signal from this spike-in control, and most of those who did were excluded during data filtering due to threshold cutoff of Cp <35. Only 9 plasma samples remained where UniSp5 might be considered for further analysis. These results indicated that careful consideration is needed particularly when suggesting specific miRNAs as potential biomarkers, since if the concentration in initial sample is low in a chosen specimen, the detection might be limited or impossible.

Based on the obtained dilution range approach, we initially eliminated two FF samples and three FFPE samples from our dataset. To address this, we prepared new cDNA from RNA stock. One sample still provided unfavorable Cp values and another extraction was performed, resulting in a full set of reliable samples for analysis. This shows the utility of the QC panels in identifying problematic samples at a relatively low cost, before running a large-scale expensive panel.

Hemolysis releases miRNAs from red blood cells and platelets and has been shown to impact the miRNA content in blood samples (30, 47, 48). In our study, we found no ΔCp(miR-451a, miR-23a) values above 7, which indicated that plasma samples are suitable for miRNA studies. Identification of stable endogenous references is crucial when analyzing RT-qPCR data. Though U6 has been reported to be variably expressed across different types of tissues, including various types of cancer (20), it has been widely used to normalize miRNA expression in OC tissues and cells (24, 25, 49). The use of U6 seems to originate from very few studies, none of which concerned ovarian tissues (50, 51).

To our knowledge, only a few studies have so far been published on the identification of reliable endogenous miRNA controls in EOCs (23, 52, 53). The algorithms used to identify the most stable candidates for normalization frequently rank the candidates differently, leading to diverse results based on the approach undertaken by different research teams (54) (Figure 5 and Table IV). Among endogenous stable miRNA candidates in OC, hsa-miR-103a-3p and hsa-miR-191-5p were suggested in previous studies (23, 55). Bignotti *et al*. tested the stability of eleven putative endogenous miRNA candidates on a total of 75 high-grade serous OC and normal tissues from 30 patients (15 ovarian and 15 fallopian tube) using RT-qPCR. Among these candidates, hsa-miR-191-5p and hsa-miR-103a-3p, which are also included in miRCURY LNA miRNA QC PCR Panels, were ranked differently using GeNorm and Normfinder algorithms (23). Hsa-miR-191-5p was suggested as the most suitable candidate for data normalization, which is in agreement with our observations for FF+FFPE+plasma, plasma, and FFPE, but not for FF. Wang *et al*. used the combination of hsa-miR-103a-3p and cel-miR-39 when calculating the expression of miRNAs in serum, but only U6 when assessing miRNA expression in tissues (55). In our study, hsa-miR-103a-3p was observed to be the best candidate only in the FF group. Evident discrepancies between the five algorithms used for stability ranking of controls and endogenous miRNAs were observed (Figure 5, Table IV), indicating that ample care should be taken when selecting an algorithm for identifying endogenous controls to be used for RT-qPCR normalization.

Unstable miRNAs like hsa-miR-451a, which was shown to be the least stable by almost all algorithms in both types of tissues and plasma types of specimens might be further investigated according to the individual characteristics of patients. Although patients were matched by FIGO stage, the differences in survival and age could serve as a stratification tool.

Our study has some limitations. One of them is the fact that studied endogenous miRNAs are not tailored for OC. Therefore, in the next step, an expanded panel of miRNAs could be used to investigate which miRNAs have potential to serve as endogenous controls in OC studies. However, two of the included miRNAs have been used as stable controls in previous studies with partial agreement with our results. Moreover, our workflow was based on the kits and solutions offered by one provider (Qiagen) and comparison with other manufactures may be relevant to find the best solution. Our choice was based on the widest peer-reviewed investigation of miRNA profiling platforms performed to date by Mestdagh *et al*., in which miRCURY technology from Qiagen showed superior results (28).

Conclusion

Our study provides a basis for further research into miRNA expression in high grade OC. Using QC panels and recording Cp range of isolation spike-in controls from no tissue controls, we demonstrated a method for identifying samples with abnormal Cp values that could easily be implemented in existing protocols from the manufacturer to consider differential dilution of RNA samples.

Experimental validation of stable endogenous controls should be performed for each type of tissue across different diseases. We suggest the use of RefFinders comprehensive ranking when assessing the stability of reference miRNAs.

Conflicts of Interest

The Authors declare no competing interests in relation to this study.

Authors' Contributions

J.L.J., E.V.H., C.K.H., D.V.N.P.O. designed the study. E.V.H. and C.K.H. provided the materials and data. P.H.D.P. and J.L.J. performed the data acquisition, processing and analyses. All authors interpreted the data. P.H.D.P. and J.L.J. wrote the manuscript. All Authors have reviewed the manuscript.

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