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ORIGINAL ARTICLE

miRNA Isolation from FFPET Specimen: A Technical Comparison of miRNA and Total RNA Isolation Methods

Zsófia Brigitta Nagy[1] · **Barnabás Wichmann**[2] · **Alexandra Kalmár**[1] · **Barbara Kinga Barták**[1] · **Zsolt Tulassay**[1,2] · **Béla Molnár**[1,2]

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Abstract MiRNA remain stable for detection and PCR-based amplification in FFPE tissue samples. Several miRNA extraction kits are available, however miRNA fraction, as part of total RNA can be isolated using total RNA purification methods, as well. Our primary aim was to compare four different miRNA and total RNA isolation methods from FFPE tissues. Further purposes were to evaluate quantitatively and qualitatively the yield of the isolated miRNA. MiRNAs were isolated from normal colorectal cancer FFPE specimens from the same patients. Two miRNA isolation kits (High Pure miRNA Isolation Kit, miRCURY™ RNA Isolation Kit) and two total RNA isolation kits were compared (High Pure RNA Paraffin Kit, MagNA Pure 96 Cellular RNA LV Kit). Quantity and quality were determined, expression analysis was performed by real-time PCR using qPCR Human Panel I + II (Exiqon) method detecting 742 human miRNAs in parallel. The yield of total RNA was found to be higher than miRNA purification protocols (in CRC: Ex: 0203 ± 0021 μg; HPm: 1,45 ± 0,8 μg; HPp: 21,36 ± 4,98 μg; MP: 8, 6 ± 5,1 μg). MiRNAs were detected in lower relative quantity of total RNA compared to the miRNA kits. Higher number of miRNAs could be detected by the miRNA isolation kits in comparison to the total RNA isolation methods. (Ex: 497 ± 16; HPm: 542 ± 11; HPp: 332 ± 36; MP: 295 ± 74). Colon specific miRNAs (miR-21-5p;-34-5p) give satisfying results by miRNA isolation kits. Although miRNA can be detected also after total RNA isolation methods, for reliable and reproducible miRNA expression profiling the use of miRNA isolation kits are more suitable.

Keywords MiRNA · Isolation kit · Comparison · Reference MiRNA · U6 · Normalization · Total RNA · Isolation · Ffpe · Colon · Colorectal cancer · Concentration

Introduction

MiRNAs are a class of short (around 19–21 nucleotides in length) non-coding RNA molecules, that endogenously regulate gene expression through post-transcriptional modification of mRNAs. More than 30 % of protein coding genes are targeted by microRNAs [1]. As miRNAs are playing a key role in cancer development many studies focusing on miRNA expression profiling. [2–4].

Clinico-pathological features of formalin-fixed, paraffin-embedded (FFPE) tissues are well documented by expert pathologists. Our group has already examined the efficiency of DNA isolation kits in case of FFPE samples. Manual and automated purification protocols were compared and it was found that DNA yield was lower in FFPE tissues in comparison to fresh frozen tissues [5]. The applied DNA isolation techniques were performed manually by QIAamp DNA Mini Kit and DNA FFPET Kit (Qiagen, Germany), automated DNA isolation was performed with MagNA Pure DNA and Viral NA SV Kit on MagNA Pure 96 System (Roche, Germany).

✉ Zsófia Brigitta Nagy
nagyzsofiab@gmail.com

[1] Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi Str. 46, Budapest 1088, Hungary

[2] Molecular Medicine Research Unit, Hungarian Academy of Sciences, Budapest, Hungary

RNA expression profiling from FFPE tissues has proven to be difficult and the obtained results are contradictory, caused by partly degraded RNA [6, 7]. Kalmár et al. had conducted a study on total RNA isolation kits testing the RNA content between matched fresh frozen and FFPE samples. Gene expression analysis of colorectal cancer specific markers was also performed in order to investigate the differences between isolation protocols and tissue types [8]. Furthermore, Li et al. found that there is a good correlation in miRNA expression pattern between FFPE cells and snap frozen cells. Isolated miRNA was analyzed by real-time PCR, where the expression of 160 miRNA were compared. [9]. It is already proved that miRNAs can be isolated from FFPE tissues successfully [10]. Furthermore, the co-isolation of miRNA with mRNA from one biological sample may allow parallel determination of miRNA expression changes with their downstream RNA target alterations.

Due to the fact that isolation methods might have impact on downstream results [11–13], this study aimed to compare four commercially available RNA isolation kits: two microRNA and two total RNA purification methods. We are interested whether total RNA isolation kits are capable to bind short RNAs such as miRNAs. We evaluated an automatic total RNA isolation method on the MagNA Pure System with the ready-to-use MagNA Pure 96 Cellular RNA LV Kit (Roche, Germany). All of used microRNA and one of total RNA isolation methods are based on silica-gel column technology. In case of the latter one, a purification robot was used to isolate total RNA with automated magnetic glass particle technology. After qualitative and quantitative evaluations, isolated RNA molecules were analyzed by miRNA expression panels consecutively. The qRT-PCR conditions, Cp values, and absolute expressed miRNA numbers were tested and compared.

Materials and Methods

1.1. Sample Collection

Formalin-fixed, paraffin-embedded (FFPE) surgically removed tissues were analyzed; and clinical data was obtained. Tissue blocks were selected that were collected less than five years ago from colorectal cancer (Dukes B CRC, $n = 3$) and normal adjacent tissue (NAT, $n = 3$) regions from the same patients. Patient age was over 18 years and mean age was 65,7 ± 7,6 years. Tumor content of each CRC block was ≥60 %; that was estimated on the basis of hematoxylin and eosin stained slides. From each FFPE block, 12 slides (thickness: 10um; 3 slides/tube) were prepared. Extractions were performed in parallel with different methods. Slides were then deparaffinized by xylene incubation twice, 10 min at room temperature each time, followed by 10-min centrifugation at 13.000 rcf. The deparaffinized tissues were incubated in absolute ethanol for 10 min at room temperature twice, then were centrifuged at 13,000 rcf for 10 min. Slides were air-dried at 55 °C for 15 min.

1.2. RNA Isolation

RNA purification methods were performed with four different methods according to the manufacturers' instructions. The High Pure RNA Paraffin Kit (Roche, Germany) and MagNA Pure 96 Cellular RNA LV Kit (Roche, Germany) are able to isolate total RNA, while the other two protocols - miRCURY™ RNA Isolation Kit, High Pure miRNA Isolation Kit - can separate small RNA, especially miRNA. MicroRNAs were isolated using the two-column protocol of the High Pure miRNA Isolation Kit resulted enriched miRNA fraction. The miRCURY™ RNA Isolation Kit – Tissue provides a method for purification of total RNA including miRNA. MagNA Pure 96 System is a robotic workstation for automated nucleic acid extraction. On-column DNase treatment was performed by using the DNase enzyme and DNase Incubation Buffer from the High Pure RNA Paraffin Kit according to the manufacturers' instructions.

1.3. Quantity and Quality Check of RNA

The concentration of RNA fraction was quantified using Qubit® RNA Assay Kit by Qubit® 1.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The Qubit fluorometer is based on fluorometric technology using fluorescent dyes emit signals only when bound to specific target molecules. It is highly selective for RNA. RNA integrity was assessed with 2100 BioAnalyzer (Agilent Technologies, Santa Clara, USA) microcapillary electrophoresis system using the Agilent RNA Pico 6000 kit according to the manufacturer's instructions. RNA integrity analyzes were performed for all isolated RNA samples, with 5 ng RNA/sample input. The 2100 Bioanalyzer makes it possible to analyze small RNA (<200 nt). RIN calculation is not reliable in case of miRNA quality analysis, as RIN number is calculated on the basis of 28S/18S ratio, however, the electropherograms in the range under 200 nucleotides can be informative about miRNA presence.

1.4. Reverse Transcription and Real-Time PCR Reaction

The miRCURY™ Universal RT microRNA PCR (Exiqon, Denmark) protocol is a two-part protocol consisting of a first-strand cDNA synthesis, followed by a real-time PCR amplification. In the reverse transcription reactions, 40 ng isolated RNA samples were used. The reverse transcription was performed according to the following protocol. In brief, poly(A) tail was added to microRNA molecules and then cDNA was synthesized. After that, the cDNA template was amplified using microRNA-specific and LNA-enhanced forward and reverse primers on the Ready-to-use microRNA PCR Human Panels I + II V2. R (Exiqon, Denmark) based on SYBR green quantification. The qPCRs were run on LC480 thermocycler (Roche, Germany) using the thermal-cycling parameters recommended by Exiqon. Each panel contains an interplate calibrator in triplicate, thus differences between plate reaction conditions can be normalized. All from the 768 wells on the panels, 742 miRNA primer sets were used to miRNA expression profiling with an exception of interplate calibrator oligos, spike-in control oligos and empty wells.

1.5. Statistical Analysis

The real-time PCR data were analyzed using LightCycler 480 Software (v.1.5). For relative quantification of gene expression U6, SNORD49A, SNORD38 reference RNA were applied as endogenous controls to compare the delta Cp values between normal and tumor groups. Hsa-miR-490-3p was also selected for normalization as it was the most stable miRNA with the lowest standard deviation on the panels between different isolations and clinical groups. The miRNA levels were calculated on the basis of ΔCp values [ΔCp = (45-Cp of target miRNA) – (45-Cp of internal reference miRNA)]. MiRNAs with Cp value lower than 40 cycles were determined as an expressed miRNA.

2. Results

2.1. RNA Quantity and Integrity of the Different Isolation Methods

Quantities of the purified RNA were measured by Qubit fluorometer. The recovery results are shown on Fig. 1. The four isolation methods resulted in different RNA yields. The recovery of High Pure RNA Paraffin Kit were found to be higher in comparison to the MagNA Pure 96 Cellular RNA LV Kit as illustrated on Fig. 1a. The recovery of miRNA isolation methods were represented in lower range compared to total RNA isolation procedures. RNA yield was higher in case of High Pure miRNA Isolation Kit compared to Exiqon method (Fig. 1b) possibly due to the fact that High Pure miRNA Isolation Kit was optimized for FFPE tissues.

The electrophoretic diagram of the isolated miRNA and total RNA samples can be seen on Fig. 2. As it was expected the miRNA isolation methods (miRCURY™ RNA Isolation Kit, High Pure miRNA Isolation Kit) resulted in short nucleotide length RNA molecules between 20 and 200 nt (Fig. 2a and c).

2.2. MiRNA Profiling on Exiqon PCR Panels

The two Exiqon panels are covering all together 742 human miRNAs. Panel I contains high-priority miRNA primer sets. These miRNAs are highly expressed generally, more likely to be differentially expressed in disease or more often cited in the literature. Panel II contains primer sets for other important but less abundant miRNAs. Our results confirm that more miRNAs were expressed on Panel I and in case of all isolation methods the number of expressed miRNAs on Panel II were found to be lower (Fig. 3).

To examine the results for all 742 miRNA on both Panels, the standard deviation of Cp values in case of High Pure miRNA Isolation Kit (HPm) versus Exiqon miRCURY™ RNA Tissue Kit (Ex) were shown to be the most uniform range. According to the scatterplot of all data, the qPCR results of the High Pure miRNA isolation method showed the highest correlation (r^2 = 0.89) compared to the other three extraction methods (Fig. 4). In general, higher Cp values were shown in case of total RNA isolation protocols that possibly indicate the lower concentration of miRNAs in total RNA extracted samples.

All the expressed miRNAs with Cp value under 40 cycles were defined as present and valid in the parallel measurements (Table 1). On the Exiqon Panels, in all 3 normal cases 57 %, (=422) in all tumor cases 72 % (=534) of the 742 miRNAs expressed after isolation by Exiqon Isolation Kit. Interestingly, with the Roche High Pure miRNA isolation method, 69 %(=511) of the miRNAs were expressed in the normal and 64 % (=474) in the cancer cases. Lower detection ratio (25–38 % = 185–281) was observed in case of total RNA Isolation Kits. Table 1 shows the detailed version of detection ratio of the analyzed samples.

2.3. Comparative Expression Analysis of Selected Colon Specific miRNA

For relative quantification of miRNA expression, reference miRNAs on Exiqon Panels were selected for comparative

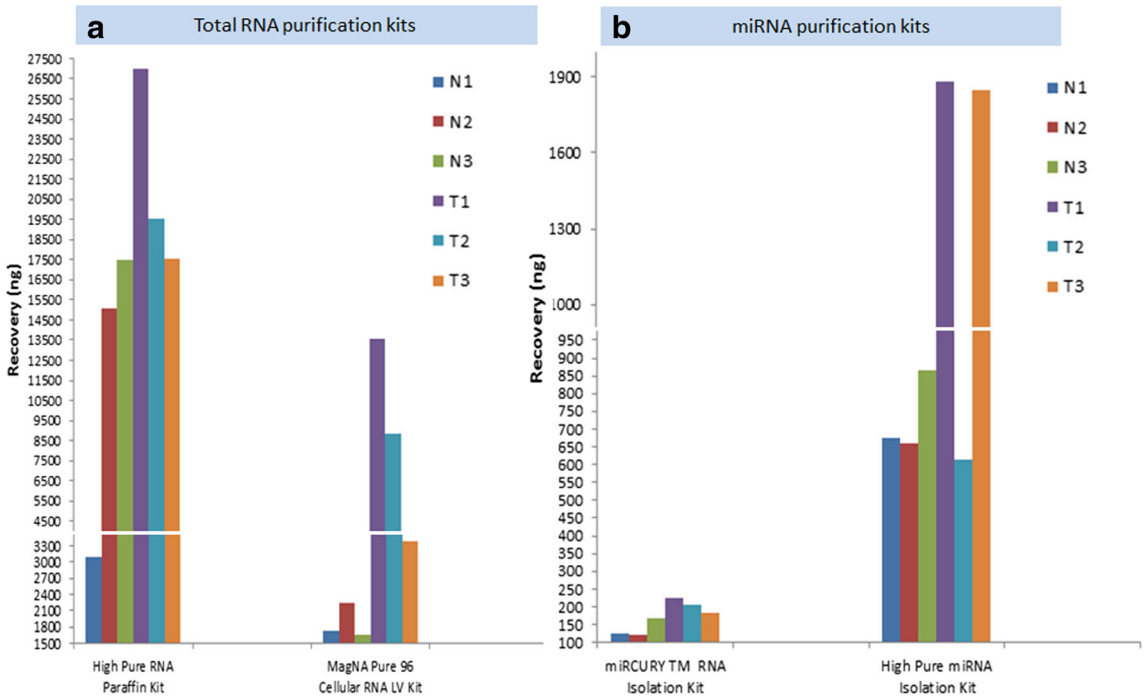

Fig. 1 Total RNA (**a**) and miRNA (**b**) levels of different isolation methods. Normal samples are represented as N1-N3 and cancer samples are T1-T3

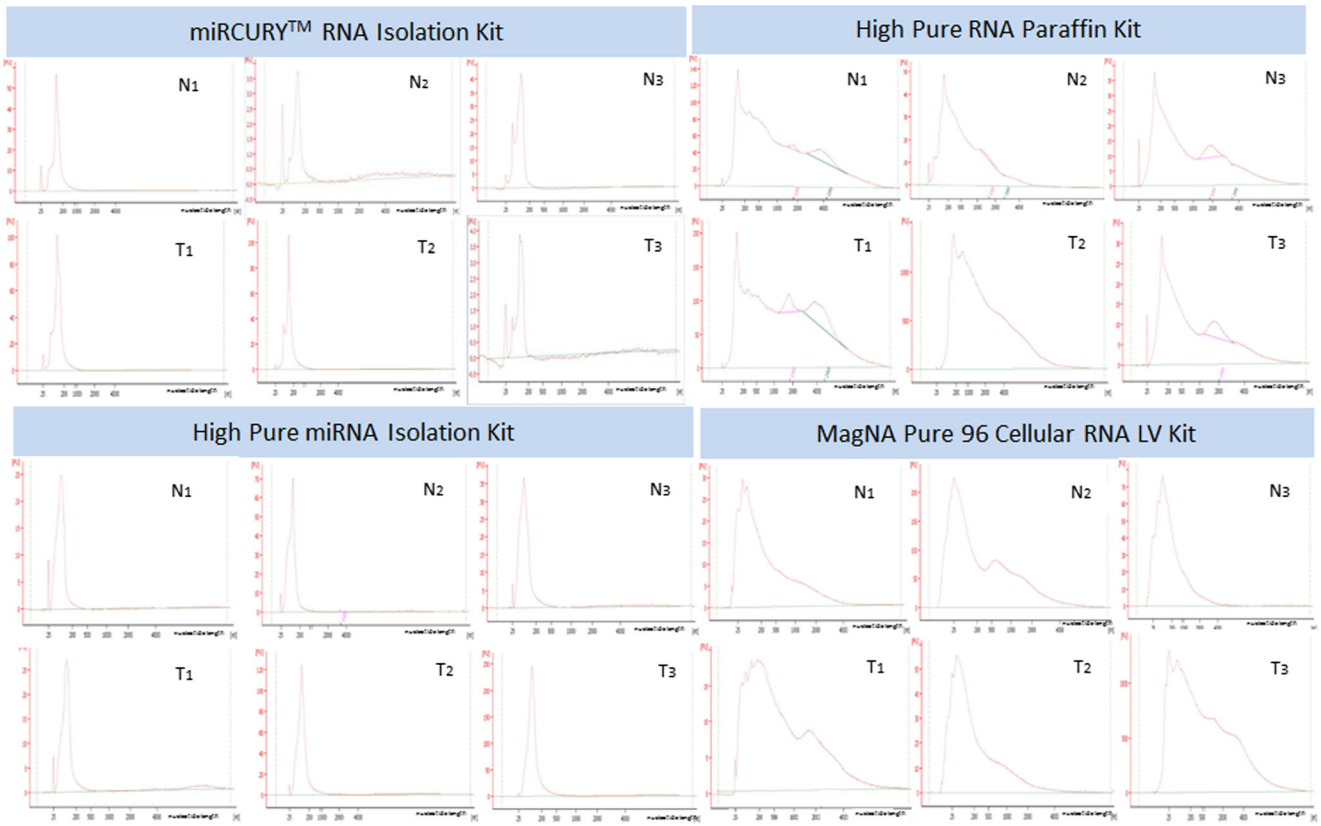

Fig. 2 Electropherogram profile of Low Molecular Weight (LMW) RNA and total RNA from four isolation methods. Normal samples are represented as N1-N3 and cancer samples are symbolized as T1-T3. Top and bottom left, figures show small RNA fragment sizes possibly containing miRNAs isolated by miRCURY RNA and High Pure miRNA Isolation Kits. Top and bottom right curves show the wide range of fragmented total RNA represented by high peaks in a small nucleotide length. The electrophoretic diagrams of MagNA Pure 96 Cellular RNA LV Kit show that the samples are more degraded, than High Pure RNA Paraffin Kit isolated samples. The small RNA fragment sizes may contain miRNA

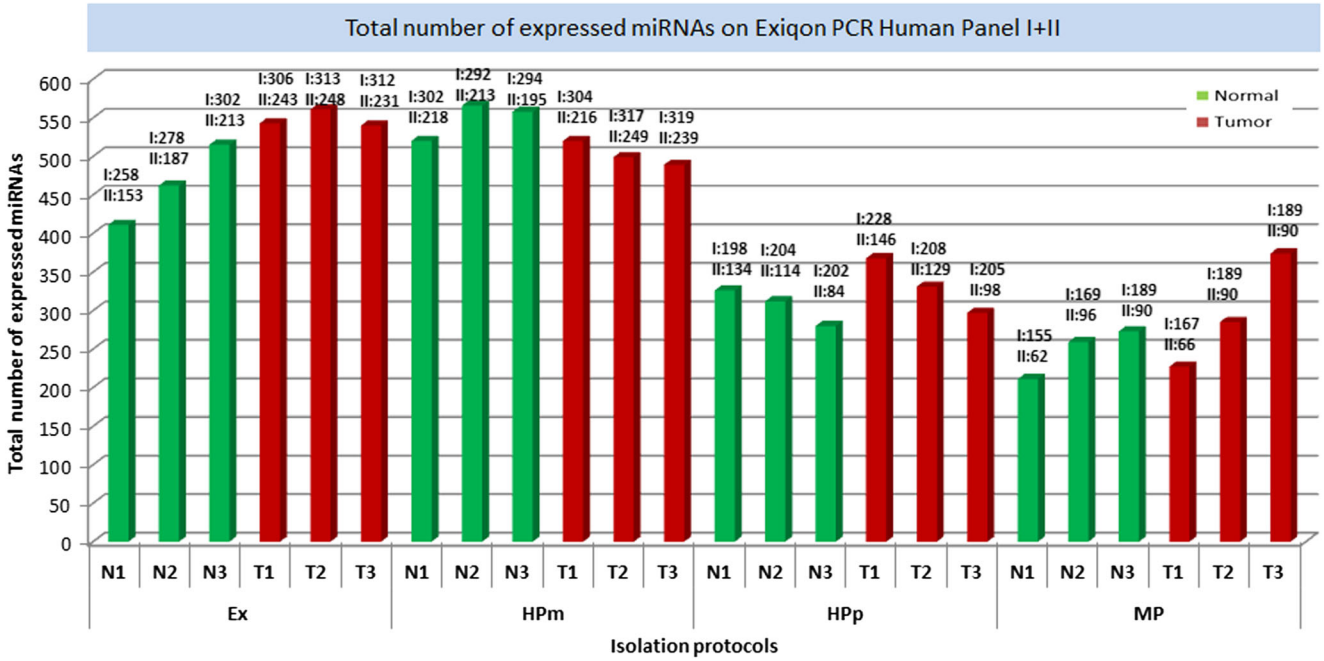

Fig. 3 Overview of expressed miRNA on Exiqon PCR Human Panel I + II in differently isolated samples. Over the bars, present numbers are detailed on Panel I and Panel II (symbolized by I and II) (HPm = High Pure miRNA Isolation Kit; Ex- Exiqon Tissue Isolation Kit; HPp = High Pure Paraffin Kit;MP = MagNA Pure 96 Cellular RNA LV Kit; N = normal T = tumor samples)

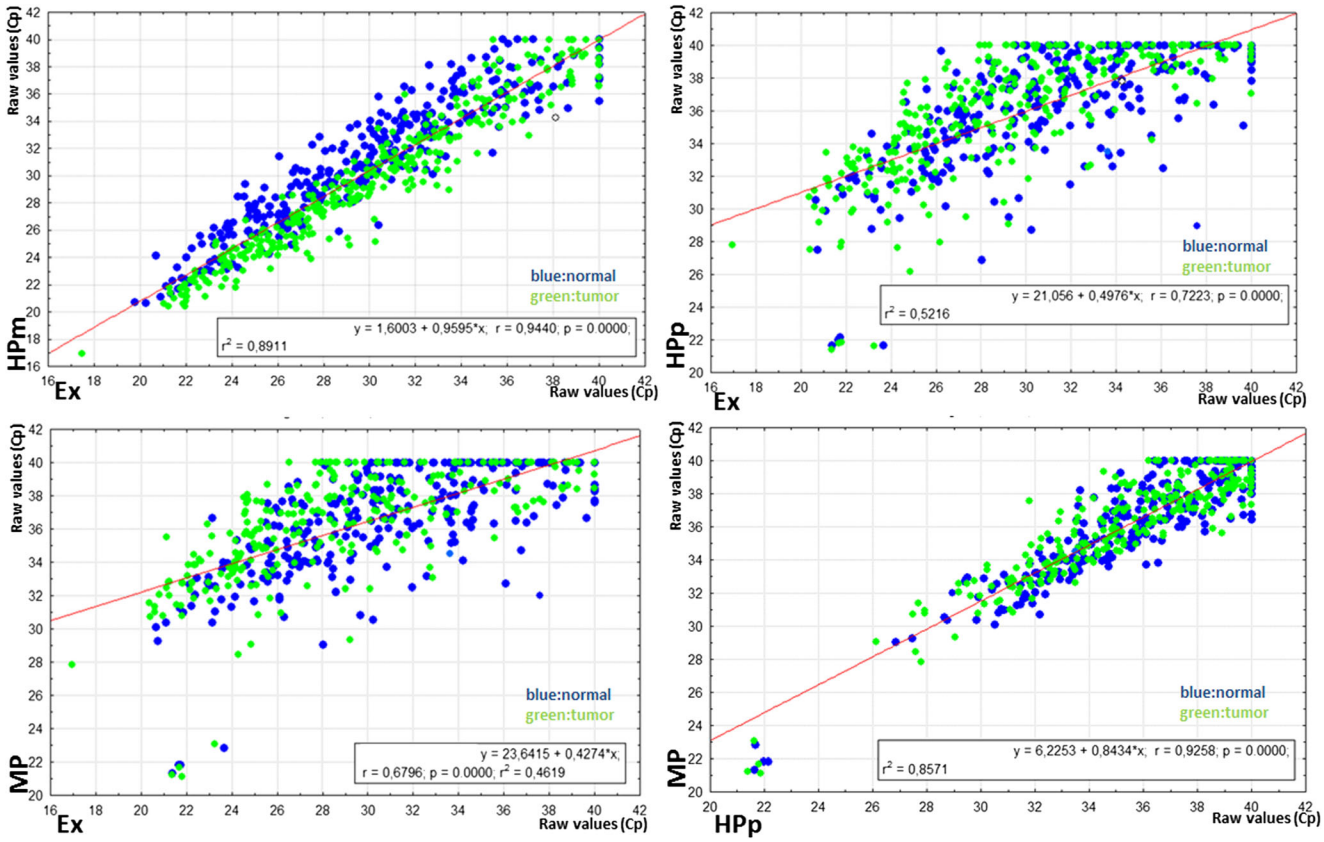

Fig. 4 Paired comparison of miRNA raw values detected by real-time PCR from different isolation protocol. Real-time PCR raw values are showing the ratio of Cp values determined by isolation methods. Colors of the dots indicate the tissue types: green = tumor sample, blue = normal sample. (HPm = High Pure miRNA Isolation Kit; Ex- Exiqon Tissue Isolation Kit; HPp = High Pure Paraffin Kit;MP = MagNA Pure 96 Cellular RNA LV Kit)

Table 1 Overview of expressed miRNAs in parallel measurements

	HPm		Ex		HPp		MP	
	N	T	N	T	N	T	N	T
Expressed miRNA in all 3 measurements	69 %	64 %	57 %	72 %	28 %	38 %	25 %	32 %
Expressed miRNA in 2 measurements	11 %	9 %	11 %	8 %	11 %	15 %	14 %	21 %
Expressed miRNA only in 1 measurements	9 %	10 %	13 %	9 %	16 %	20 %	24 %	21 %
No signal	11 %	17 %	19 %	12 %	46 %	27 %	37 %	26 %

HPm = High Pure miRNA Isolation Kit; Ex- Exiqon Tissue Isolation Kit; HPp = High Pure Paraffin Kit;MP = MagNA Pure 96 Cellular RNA LV Kit; N = normal T = tumor samples

analysis. Table 2 shows the average Cp values of 3 parallel measurements in case of three reference miRNAs (U6, SNORD38B, SNORD49A) and the selected miR-490-3p. Reference U6 was not stable in all cases and not in all methods. Nevertheless, SNORD38B, SNORD49A could be detected in all parallels and in all isolations. One more miRNA, miR-490-3p was also selected as the most stable miRNA on the panels (Table 2).

To investigate the question, whether selection of different miRNA for normalization steps have an influence to the miRNA expression between clinical groups, two individual colorectal cancer-specific miRNA were selected based on the literature references to evaluate the expression differences resulting from purification variations and housekeeping reference genes. Previous studies showed that expression of miR-21 is upregulated in CRC tumor tissues [14–16] and miR-34 family members are also expressed in gastrointestinal tissues and in human colon cancer cells [17, 18]. Raw values of miR-21 and miR-34 were normalized with a reference short RNA (SNORD38B) and with our dedicated miR-490-3p. The raw Cp values of miRNAs isolated by miRNA purification protocols were significantly lower, thus showed stronger signals than Cp values of miRNAs resulted by total RNA isolation methods. Therefore, the dCp values in case of miRNA isolation protocols are in a different range compare to the total RNA methods (Fig. 5).

On the other hand, dCp differences between clinical groups were altered among the different normalization methods. On the basis of normalization with miR-490-3p, miR-21 and miR-34 were upregulated in tumor samples after all isolation methods. These tendencies were less pronounced, if normalization was performed with SNORD38B (Fig. 5).

3. Discussion

It is of interest to develop nucleic acid isolation methods that might be utilized in diagnostic workflows in the future starting from widely available tissue samples as FFPE blocks. It is a fact that miRNAs can be successfully isolated from FFPE tissues, meanwhile it would be a challenge to purify target mRNA molecules from the same tissue with the same conditions with a combined isolation method.

In the present study four commercial isolation methods were compared in terms of miRNA yield and quality. Furthermore, in order to test the downstream applicability of the differently isolated samples RT- PCR expression analysis was performed on Exiqon Human Panel I + II v2. R platform. This method can detect more than 700 miRNAs already associated with cancer development.

The RNA yield was higher in case of High Pure miRNA Isolation Kit compared to the Exiqon method. Especially, looking on the normal cases, the number of expressed genes was higher in the High Pure miRNA purification method, where the isolated amount of miRNA was significantly lower with the Exiqon kit. The total RNA recovery of the High Pure RNA Paraffin Kit was found to be higher in comparison to MagNA Pure 96 Cellular RNA LV Kit.

In several studies the concentration of miRNAs is measured by spectrophotometer [19, 20]. However, due to the low concentration of miRNA fraction in tissue samples it is not a reliable miRNA quantification method and because of the possible overestimation of the miRNA quantity due to DNA and RNA contamination.

Quality results showed that miRNA fraction was successfully extracted by High Pure miRNA isolation Kit and Exiqon miRCURY™ RNA Isolation Kit. Electrophoretic diagrams also confirmed small RNA content, because characteristic peaks were detected in the range of nucleotide length of miRNAs (200 nt). The presence of miRNA is presumable also after total RNA isolation methods on the basis of microcapillary electrophoresis results as a confirmation, however, as a confirmation, remarkable number of miRNAs could be detected also by qPCR analysis.

Jung et al. described that mRNA degradation is not accompanied with miRNA destabilization and RIN values do not

Table 2 Overview of average Cp value of reference miRNAs. Accepted reference miRNAs were provided on Panels and the most stable miR-490-3p were also selected and described in the table

Purification protocols	HPm				Ex				HPp				MP			
Sample types	N		T		N		T		N		T		N		T	
Reference miRNA	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value	detection of 3 parallels	avg.Cp value
U6	2/3	34,65 ± 0,1	2/3	35,74 ± 1,2	0/3	NA	1/3	NA	2/3	29,44 ± 0,9	3/3	28,96 ± 1,3	3/3	32,75 ± 3,4	3/3	32,01 ± 1,8
SNORD38B	3/3	28,61 ± 0,3	3/3	29,82 ± 0,9	3/3	28,03 ± 1,1	3/3	24,86 ± 0,8	3/3	26,85 ± 0,3	3/3	26,15 ± 0,5	3/3	29,1 ± 0,2	3/3	29,08 ± 1,0
SNORD49A	3/3	25,52 ± 0,7	3/3	27,15 ± 1,1	3/3	30,24 ± 2,2	3/3	26,18 ± 1,7	3/3	28,68 ± 0,2	3/3	27,94 ± 1,0	3/3	30,56 ± 0,5	3/3	30,93 ± 1,9
hsa-miR-490-3p	3/3	32,16 ± 0,5	3/3	31,6 ± 1,6	3/3	32,62 ± 1,8	3/3	32,85 ± 0,2	3/3	32,59 ± 0,8	3/3	32,67 ± 0,2	3/3	33,17 ± 0,7	3/3	33,04 ± 0,5

HPm = High Pure miRNA Isolation Kit; Ex- Exiqon Tissue Isolation Kit; HPp = High Pure Paraffin Kit;MP = MagNA Pure 96 Cellular RNA LV Kit; N = normal T = tumor samples

have negative effect on miRNA in renal and prostate samples resulted by real-time PCR method [20]. Our results showed that miRNAs were detected by lower RIN values in case of miRNA isolation protocols and in total RNA isolation protocols too, limited detectable miRNAs were found in total RNA Isolation kits compared to miRNA isolation protocols. MiRNAs can be analyzed from degraded RNA samples as a result of their stable form [20].

Nucleic acid co-isolation could be advantageous and combined methods are already available, as Kotorashvili et al. proved that co-extraction of DNA and RNA from FFPE tissues are highly efficient [21]. Our investigation to extract miRNA with total RNA isolation kits was partially successful with High Pure RNA Paraffin Kit and MagNA Pure 96 Cellular RNA LV Kit. The FFPE samples yielded enough total RNA and small RNA fragment sizes containing miRNAs that could be detected by qRT-PCR.

Minimally altered expression signals were observed between the Exiqon and High Pure miRNA Isolation Kit, expression level alteration tendencies of the detected miRNAs were similar. According to the results of Li et al., robust miRNA profile was detected in FFPE cells extracted by total RNA isolation kit [9]. In our results less miRNA were found to be expressed using total RNA isolation kits than with the miRNA isolation protocols.

By comparing the real-time PCR results after the four isolation methods, in general, the use of miRNA isolation kits resulted in higher number of expressed miRNAs on both Panels compared to the total RNA isolation methods. The High Pure miRNA Isolation Kit showed the highest number of the expressed miRNAs on both Panels (Fig. 3). In contrast, total RNA isolation kits showed higher failed/successful ratio of RT-qPCR reactions (Table 1).

In order to analyze the isolated miRNA samples in downstream analysis two colorectal cancer specific miRNAs (miR-21 and −34-5p) were selected to be analyzed quantitatively. Therefore reference miRNA for normalization was selected. The selection of proper reference miRNA is critical in miRNA expression studies, as miRNA expression results largely depend on the applied reference miRNA applied for normalization.

Although, miRNA U6 which is a generally accepted housekeeping reference gene, it was not available for normalization in our experiments due to the lack of data in case of some samples. As housekeeping gene, two other standard genes the SNORD 38 and -49A have shown relatively high standard deviation (Table 2). Due to the above-mentioned reasons we aimed to select new reference miRNA.

MiR-21 was selected to the basis of its upregulation in tumor samples according to previous microarray and real-

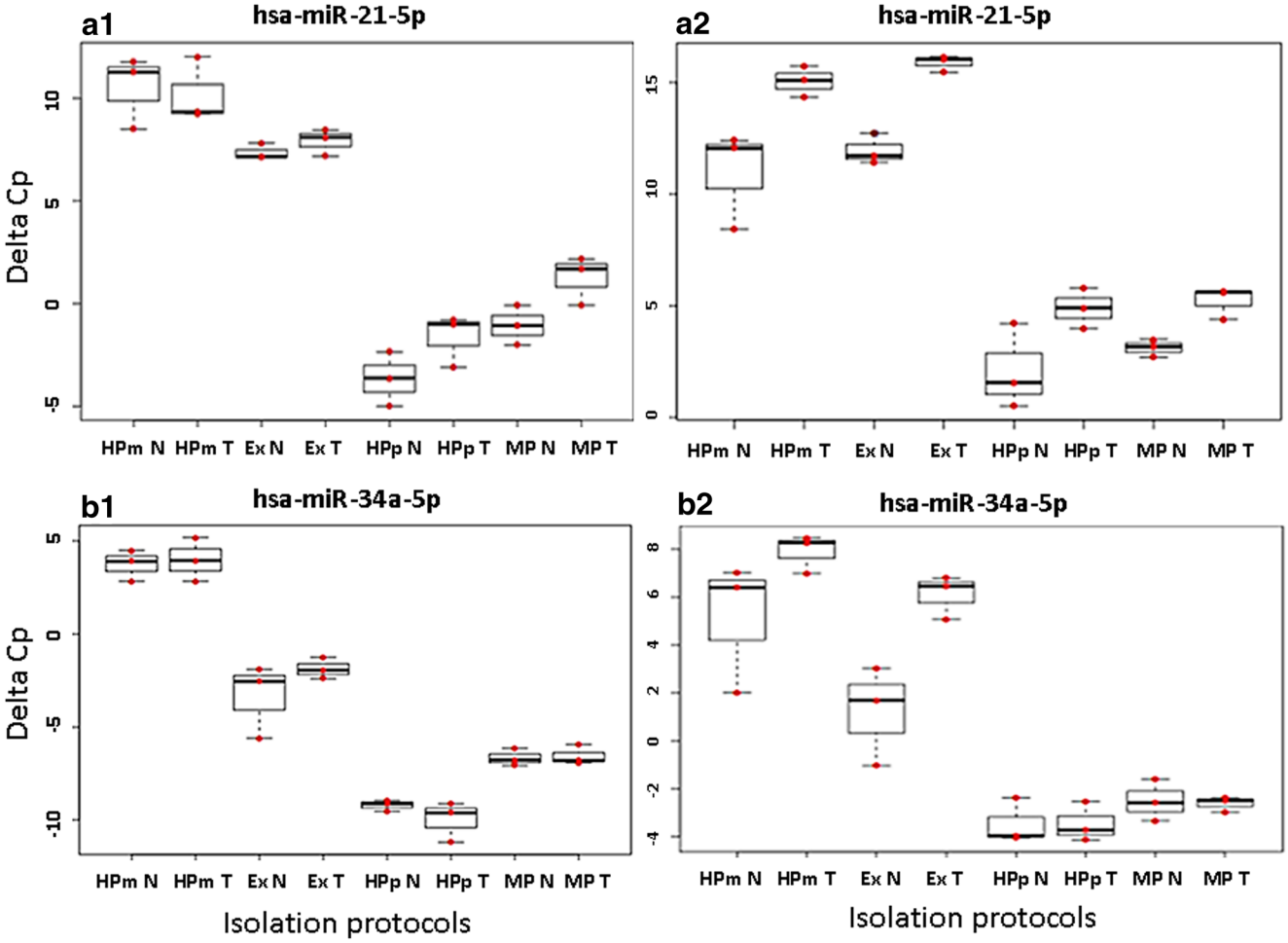

Fig. 5 Boxplot visualization of deltaCp (cycle threshold) values for two selected colorectal cancer-related miRNAs from different isolation methods. Boxplot A1-B1 show the dCp with normalization by SNORD38B, boxplot A2-B2 show the dCp values normalized by hsa-miR-490-3p. Raw Cp values were extracted from the maximum 45 cycles. (HPm = High Pure miRNA Isolation Kit; Ex- Exiqon Tissue Isolation Kit; HPp = High Pure Paraffin Kit;MP = MagNA Pure 96 Cellular RNA LV Kit)

time PCR results [16]. The upregulation of miR-21 was confirmed by our results after normalization with our selected reference miR-490-3p. SNORD 38B is a small nucleolar RNA, also known as U38B or RNU38B. It has been used as a reference miRNA in several studies [22, 23]. If raw values were normalized by SNORD38B, differences between normal and CRC samples were less pronounced.

Another miRNA, the miR-34-5p, was selected to further investigations. According to previous studies, Gao et al. proved miR-34a-5p down regulation in primary CRC tumors [24]. Furthermore, Hiyoshi et al. reported that miR-34 family members are significantly increased in colon tumors [18]. Our results of the selected reference miRNA are in line with the literature.

In summary, four different RNA isolation protocols were tested on FFPE tissues. After quantity measurements, miRNA content was examined by real-time PCR method on a ready-to-use plate system. miRNAs were successfully detected after all four purification methods, although miRNA specific isolation protocols showed higher miRNA yield than total RNA extraction kits. Our investigation suggest that the selection of reference miRNA is critical in order to perform reliable miRNA expression profile analysis.

Acknowledgement

Conflict of Interest The Authors declare that there is no conflict of interest.

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