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Optimized procedure for high-throughput transcriptome profiling of small extracellular vesicles isolated from low volume serum samples

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Abstract

Objectives: Small extracellular vesicles (EVs) contain various signaling molecules, thus playing a crucial role in cell-to-cell communication and emerging as a promising source of biomarkers. However, the lack of standardized procedures impedes their translation to clinical practice. Thus, we compared different approaches for high-throughput analysis of small EVs transcriptome.

Methods: Small EVs were isolated from 150 µL of serum. Quality and quantity were assessed by dynamic light scattering, transmission electron microscopy, and Western blot. Comparison of RNA extraction efficiency was performed, and expression of selected genes was analyzed by RT-qPCR. Whole transcriptome analysis was done using microarrays.

Results: Obtained data confirmed the suitability of size exclusion chromatography for isolation of small EVs. Analyses of gene expression showed the best results in case of samples isolated by Monarch Total RNA Miniprep Kit. Totally, 7,182 transcripts were identified to be deregulated between colorectal cancer patients and healthy controls. The majority of them were non-coding RNAs with more than 70 % being lncRNAs, while protein-coding genes represented the second most common gene biotype.

Conclusions: We have optimized the protocol for isolation of small EVs and their RNA from low volume of sera and confirmed the suitability of Clariom D Pico Assays for transcriptome profiling.

Keywords: colorectal cancer; high-throughput expression profiling; long non-coding RNAs; size exclusion chromatography; small extracellular vesicles; transcriptome

Introduction

Over the last years, secreted membrane-enclosed vesicles are being intensively studied. The best described are exosomes, small extracellular vesicles (EVs) of endocytic origin with size ranging from 30 to 150 nm [1]. Initially, they were considered to be an unfunctional garbage bags. However, it was proved that they contain variety of molecules such as nucleic acids, lipids, proteins, or different metabolites [2], facilitate the transport of these intracellular components, and thus play a crucial role in cell-to-cell communication [3]. Importantly, recent studies have demonstrated their active release into the peripheral circulation by cancerous cells [4], thus they have emerged as a promising source of novel non-invasive biomarkers for early cancer diagnosis, prognosis and therapy response monitoring [5].

Despite the growing number of studies describing circulating small EVs as a promising liquid biopsy, only few biomarkers associated with EVs have entered the clinical trials [6, 7] due to the lack of standardized procedures. Although the ultracentrifugation is the most widely used method [8], it is time-consuming, requires the right equipment, and usually higher sample volume. Moreover, shearing forces may cause the disruption of vesicles and its efficiency is reduced when high viscosity fluids such as

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serum or plasma are used [9]. Therefore, numerous commercial kits have been developed to improve and facilitate isolation of EVs. These methods are based on precipitation, ultrafiltration, size-exclusion chromatography (SEC), or immunoassays [10]. Compared to the centrifugation, the structure of vesicles is usually not changed, low volumes of sample are required, different eluting solutions may be used, and in case of immunoassays, highly pure fraction of small EVs may be acquired. Nevertheless, precipitation reagents inhibit recovery of intact EVs and polymers could interfere with downstream analysis and influence the biological activities [11, 12]. Similarly, ultrafiltration methods have been shown to result in low purity and adhesion of EVs to the filtration membrane, while SEC requires longer running time and vesicles are eluted in a larger volume, thus additional concentration step is necessary. Finally, immunoassays are expensive and usually used for low volumes of highly concentrated samples. In addition, non-specific binding was observed, and the yield is relatively small to be sufficient for majority of further analysis [13].

Once small EVs have been isolated, their comprehensive characterization must be performed. According to the International Society for Extracellular Vesicles (ISEV), single vesicles should be characterized by at least two different technologies such as transmission electron microscopy (TEM), atomic force microscopy (AFM), nanoparticle-tracking analysis (NTA), resistive pulse sensing (RPS), or dynamic light scattering (DLS). In addition, the amount of three or more proteins from different categories that are expected to be present in small EVs should be assessed by Western blot (WB), flow cytometry, or global proteomic analysis [14]. Importantly, the level of other proteins not expected to be enriched in vesicles of endosomal origin should be determined in order to reveal the co-isolation of EVs of different origin [14].

Further, RNA extraction together with the measurements of its quality and quantity is a crucial step before the high-throughput screening of RNA content of EVs. Currently, a number of conventional RNA extraction methods are available. However, previous studies showed that different isolation methods give extensive variation in RNA yield, pattern, and purity [15, 16]. Thus, it is necessary to choose the best approach according to the aims of the research. Due to the low levels of RNA in EVs, its quality and quantity is usually assessed using Agilent 2100 Bioanalyzer together with an RNA 6000 Pico Kit [16]. Finally, identification of small EVs RNA-based biomarkers may be performed using several different methods, including RT-qPCR, digital PCR or high-throughput approaches such as microarrays or next-generation sequencing (NGS). While microarray procedure is simple and requires lower sample volume, it is prone to DNA contamination and enable detection of only known RNA sequences. On the contrary, NGS is time-consuming and its results have to be validated, but detection of different types of RNA may be performed in the same run independently of previous sequence information and together with existing isoforms [13, 17].

To date, several studies have compared the effect of different techniques for isolation of small EVs and their RNA on efficiency, reproducibility, and downstream applications [16, 18–28]. Nevertheless, none of these papers have performed in depth analyses of all important parameters. Thus, the aim of this study was to compare several techniques widely recommended for the isolation of small EVs, their characterization, and subsequent RNA isolation together with quality and quantity assessments. Further, we hypothesize that expression profiles of small EVs could differentiate between CRC patients and healthy controls. Thus, Human Clariom D Pico Assays have been tested in order to confirm its suitability for transcriptome profiling of small EVs isolated from minimal volume of blood serum as this product should be able to generate robust expression profiles data from as little as 100 pg of total RNA. To our knowledge, this is the first study comparing and optimizing different methodological approaches for credible analysis of small EVs-enriched RNAs from low volume of sera using microarrays.

Materials and methods

Patients' samples and study design

Optimization of small EVs isolation as well as RNA was performed using serum samples of healthy controls. Six serum samples of histopathologically verified CRC patients (three men, three women; three C18, three C20; 2× stage I, 1× stage II, 2× stage III, 1× stage IV; median age 63 years) and six samples of healthy controls (three men, three women; median age 68 years) were used for the expression profiling by Clariom D Pico Assays (Applied Biosystems, Waltham, MA, USA). All patients underwent the resection from 2016 through 2020 at Masaryk Memorial Cancer Institute (MMCI, Brno, Czech Republic) and samples were collected prior the surgery. Serum samples from healthy controls were collected at the Cancer Prevention Center between 2018 and 2021 (MMCI, Brno, Czech Republic); these donors had no prior diagnosis of any malignancy. All serum samples have been stored at −80 °C before the exosome isolation.

Isolation of small extracellular vesicles

Isolation of small EVs was performed using miRcury Exosome Isolation Kit – Serum and Plasma (Exiqon, Vedbaek, Denmark), Plasma/Serum Exosome Purification Mini Kit (Norgen Biotek, Thorold, Canada), and qEVsingle/35 nm columns (SP6, iZON Science Ltd., Oxford, UK). All samples were centrifuged at 1,500×*g* for 10 min at 4 °C and then at 10,000×*g* for 20 min at 4 °C. Small EVs were isolated according to the

manufacturer's recommendations. In case of SEC, individual fractions were collected (each 200 µL). Subsequently, fractions six and seven were combined. All samples were treated with 10 µL of proteinase K (20 mg/mL) and 5 µL of RNase Cocktail Enzyme Mix (20,000 U/mL) at 37 °C for 10 min. Finally, 4 µL of SUPERaseIn RNase Inhibitor (20 U/µL) were added and mixed well (all Invitrogen, Waltham, MA, USA).

Dynamic light scattering

Fifty microliters of the sample were placed in low volume quartz batch cuvette ZEN2112 (minimal volume 12 µL of sample for back scatter, 20 µL for side scatter, and 45 µL for forward scatter measurements) and measured using multi-angled DLS technique (MADLS®), Zetasizer Ultra at a constant temperature of 25 °C. The data were collected at three angles, 173°, 90°, and 13° and evaluated using ZS Xplorer software version 2.50 (all Malvern Panalytical Ltd, UK).

Transmission electron microscopy

Four microliters of the sample were applied to freshly plasma-cleaned TEM grids with thin carbon layer and stained with 2 % uranyl acetate. The grids were loaded to Talos F200C TEM (Thermo Fisher Scientific, Brno, Czech Republic). The microscope was operated at 200 kV. The images were collected on Ceta-16 M CMOS camera at the 36,000× nominal magnification with the underfocus of 2–4 µm.

Protein analysis by Western blot

All samples were concentrated to 80 µL using Concentrator plus 5305 Vacuum Centrifuge (Eppendorf, Hamburg, Germany). Protein quantification was performed using the Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 8 µg of proteins were loaded per lane. Proteins were resolved by 10 % SDS-PAGE gel and wet-transferred to Immobilion-P PVDF membrane (EMD Millipore, Burlington, MA, USA). The signals were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and detected by the UVITEC chemiluminescence imager (UVITEC Cambridge, UK). A list of antibodies is summarized in Supplementary Table S1.

Isolation of RNA

Three different kits were compared including MagMAX *mir*Vana Total RNA Isolation Kit (Applied Biosystems, Waltham, MA, USA), Plasma/Serum RNA Purification Mini Kit (Norgen Biotek, Thorold, Canada), and Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA). Total RNA including small RNAs was isolated according to the manufacturer's recommendations, treated with DNase I, and eluted with 50 µL of DEPC-treated water.

Reverse transcription and RT-qPCR

Complementary DNA was synthesized using 5 µL of total RNA and the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's recommendations. A preamplification step was performed mixing 2.5 µL of cDNA with pooled TaqMan assays (0.2×) and TaqMan PreAmp Master Mix using 14 cycles of preamplification. Quantitative PCR was carried out using specific probes (Supplementary Table S2), 2.5 µL of preampliefied cDNA diluted 20× and TaqMan Gene Expression Master Mix, fluorescence was measured by QuantStudio 12K Flex Real-Time PCR system (all Applied Biosystems, Waltham, MA, USA).

Microarray analysis

Microarray analysis was performed using Clariom D Pico Assay, human (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's recommendations. The pre-IVT amplification consisted of 12 cycles. Arrays were stained using an Affymetrix GeneChip Fluidics Station 450 according to the FS450_0001 protocol and scanned with an Affymetrix GeneChip Scanner 3000 7G.

Statistical analysis

Quality control check was done using the 'arrayQualityMetrics' [29]. To have a full control over the process of gene annotation, we created our own CDF using probe sequences from BRAINARRAY CDF [30] and the GENCODE (v40) database as a target. The 25 bp long probe sequences were aligned using BLASTn [31]. Promiscuous probes mapping to multiple different genes were removed. Only probe sets of more than four probes were allowed. Previously published script [32] was applied to build our own custom CDF and then the 'pdInfoBuilder' was used to create the R package. The differential expression analysis was done employing the 'oligo' and 'limma' packages. The expression values were normalized by RMA algorithm and p-values were adjusted by Benjamini–Hochberg.

Results

Identification of the best approach for isolation of small extracellular vesicles

To choose the best approach for the isolation of small EVs from limited volume of human blood serum, we compared qEVsingle/35 nm columns from iZON, Plasma/Serum Exosome Purification Mini Kit from Norgen Biotek, and miRCURY Exosome Serum/Plasma Kit from Exiqon. In all cases, small EVs were isolated from 150 µL of serum and their quality and quantity was assessed using DLS and TEM. In case of SEC, nine different fractions (F1–F9) were collected and separately analyzed to identify fractions with the highest amount and quality of vesicles. In addition, protein content-based characterization of gained particles was performed by Western blot according to ISEV recommendations [33] to demonstrate the degree of purity of isolates.

TEM of samples isolated by kits from Norgen Biotek and Exiqon showed a considerable number of smaller particles as well as proteins (Figure 1B and C). DLS analysis corresponded to the results of TEM and showed high polydispersity index (PDI) values for these samples indicating the

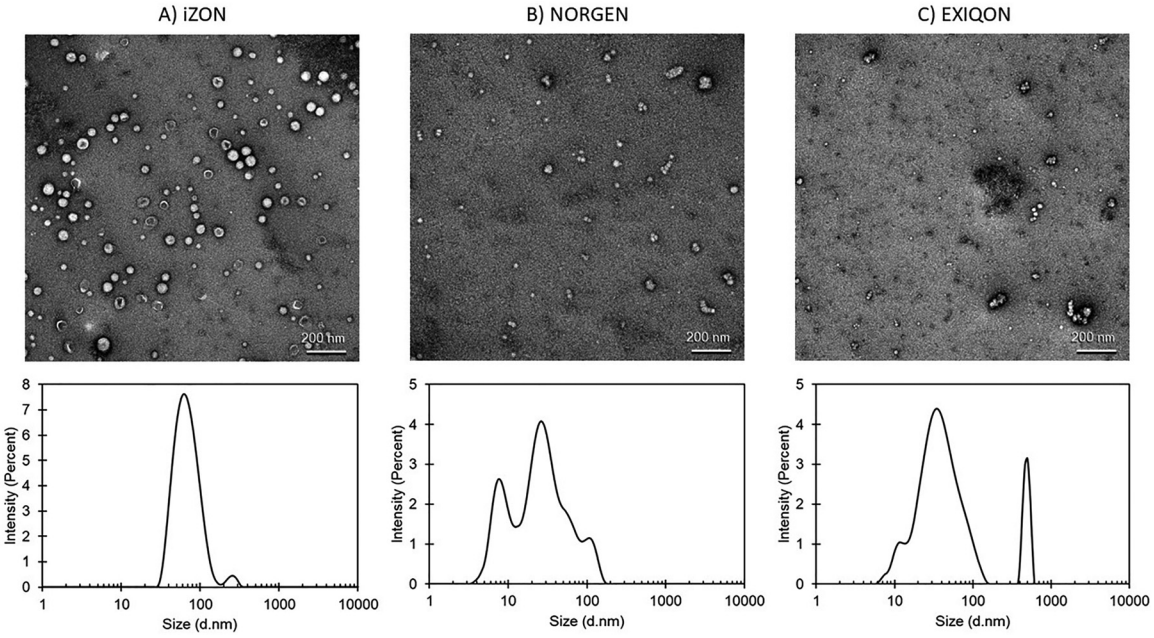

Figure 1: Transmission electron microscopy and dynamic light scattering analyses of small extracellular vesicles isolated by (A) qEVsingle/35 nm columns from iZON (fraction 6+7), (B) Plasma/Serum Exosome Purification Mini Kit from Norgen Biotek, and (C) miRCURY Exosome Serum/Plasma Kit from Exiqon.

Table 1: Results of DLS analyses of three independent samples of small extracellular vesicles isolated from serum of healthy controls by three different approaches.

Sample	Z ± SD, nm	Z-average ± SD, nm	PDI ± SD	PDI-average ± SD	c ± SD, particles/mL	c-average ± SD, particles/mL
iZON 1[a]	72 ± 1	70 ± 4	0.268 ± 0.005	0.234 ± 0.032	$9.3 \times 10^{12} \pm 2.8 \times 10^{12}$	$3.4 \times 10^{13} \pm 4.1 \times 10^{13}$
iZON 2[a]	66 ± 0		0.205 ± 0.010		$8.1 \times 10^{13} \pm 8.5 \times 10^{13}$	
iZON 3[a]	72 ± 12		0.229 ± 0.034		$1.2 \times 10^{13} \pm 2.5 \times 10^{12}$	
Norgen 1[b]	25 ± 0	26 ± 6	0.465 ± 0.018	0.446 ± 0.023	$1.0 \times 10^{18} \pm 3.3 \times 10^{17}$	$5.9 \times 10^{17} \pm 3.8 \times 10^{17}$
Norgen 2[b]	32 ± 1		0.452 ± 0.015		$2.9 \times 10^{17} \pm 1.8 \times 10^{17}$	
Norgen 3[b]	20 ± 1		0.420 ± 0.003		$4.5 \times 10^{17} \pm 7.7 \times 10^{16}$	
Exiqon 1[c]	51 ± 2	49 ± 13	0.477 ± 0.009	0.452 ± 0.023	$9.3 \times 10^{16} \pm 1.3 \times 10^{17}$	$5.2 \times 10^{16} \pm 4.1 \times 10^{16}$
Exiqon 2[c]	60 ± 1		0.447 ± 0.009		$1.0 \times 10^{16} \pm 5.5 \times 10^{15}$	
Exiqon 3[c]	35 ± 1		0.432 ± 0.038		$5.3 \times 10^{16} \pm 6.4 \times 10^{16}$	

SD, standard deviation; PDI, polydispersity index; c, concentration. [a]iZON – qEVsingle/35 nm columns from iZON (fraction 6 + 7), [b]Norgen – Plasma/Serum Exosome Purification Mini Kit from Norgen Biotek, [c]Exiqon – miRCURY Exosome Serum/Plasma Kit from Exiqon.

presence of particles with very broad size range (Table 1). In case of SEC, TEM and DLS confirmed the presence of proteins and their aggregates and almost no small EVs in fractions F1–F5, which are the part of the column initial void volume (average size of eluted particles was bigger than 300 nm). Fractions F6 and F7 contained mostly the particles with the expected size of small EVs (around 100 nm), while F8 and F9 contained a mix of particles with various sizes, but smaller vesicles predominated (Figure 2A). DLS measurements proved the highest concentration of particles in F8 and F9, however, with smaller sizes, while the vesicles in F6 and F7 contained the particles between 50 and 150 nm (Supplementary Table S3). According to the results of WB, the highest levels of small EVs markers CD81 and Syntenin were detected in F7 with lower levels in F6 and F8. Nevertheless, the amount of ApoB increased significantly from F6 to F9, thus the combination of only F6 and F7 was chosen for further experiments to minimize the contamination by lipoproteins (Figure 2B). Comparison of different isolation methods proved the expression of CD63 and TSG101 in all analyzed samples, while CD81 was detected only in samples isolated by SEC and Syntenin was not present in the samples isolated by the kit from Exiqon. Concerning the lipoprotein contamination, the purest isolates were gained using Norgen procedure, while samples isolated by Exiqon kit or SEC contained detectable amount of ApoB (Figure 2C). These data

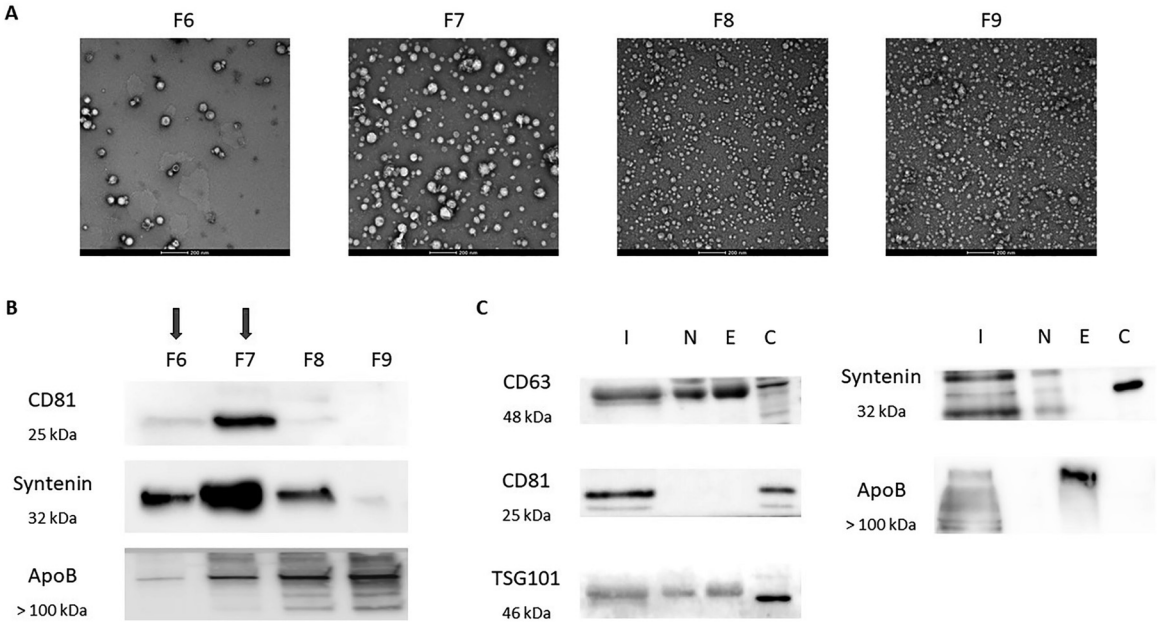

Figure 2: Transmission electron microscopy (TEM) and western blot (WB) analyses of isolated extracellular vesicles. (A) TEM of fractions F6–F9 isolated by qEVsingle/35 nm columns (iZON), (B) Detection of selected protein markers by WB in F6–F9 isolated by qEVsingle/35 nm columns (iZON), and (C) Detection of selected protein markers by WB in samples isolated by qEVsingle/35 nm columns from iZON (I), Plasma/Serum Exosome Purification Mini Kit from Norgen Biotek (N), miRCURY Exosome Serum/Plasma Kit from Exiqon (E), and in cell lysate (HCT-116 cells) (C).

confirm the suitability of qEVsingle/35 nm columns for isolation of small EVs from low volume of sera as this approach enable to get high number of vesicles with corresponding size, shape, and sufficient purity although the lipoproteins are co-isolated to some extent.

Identification of optimal procedure for the RNA isolation from small extracellular vesicles

To find the best method for the extraction of RNA from isolated small EVs, we compared three different commercial kits – MagMAX *mir*Vana Total RNA Isolation Kit (Applied Biosystems), Exosomal RNA Isolation Kit (Norgen Biotek), and Monarch® Total RNA Miniprep Kit (New England BioLabs). As the aim of the study was to further analyze the expression of lncRNAs as well as protein-coding genes by Clariom D Pico Assays, we assessed the efficiency of RNA extraction kits by RT-qPCR analyses of selected lncRNAs and protein-coding genes previously described to be present in small EVs [34–37]. In case of all genes, the lowest expression was observed in samples isolated by the kit from Norgen Biotek. The detection of all targets by two other kits was comparable, only in case of lncRNA GAS5 the detection rate was higher when isolation was performed by Monarch kit compared to MagMAX kit (Table 2). To further evaluate the suitability of RNA isolated by the selected kits for high-throughput expression profiling, we compared the microarray results from two independent samples of healthy controls. Interestingly, the global gene expression was higher in case of samples isolated by Norgen Biotek kit compared to the MagMAX isolation kit and comparable to the results of arrays using samples isolated by Monarch kit (Supplementary Figure S1). Based on these data, we decided to use Monarch Total RNA Miniprep Kit for further isolation of RNA as the detection rate of all genes was the highest in case of RT-qPCR as well as in microarray analyses.

Expression profiling of serum-derived small extracellular vesicles using Clariom D Pico Assays

Based on the results of the optimization phase of the study, we decided to use qEV single/35 nm columns from iZON for exosome isolation from sera, while RNA was isolated using the Monarch Total RNA Miniprep Kit. To assess the suitability of such isolated RNA for high-throughput screening of lncRNAs as well as protein-coding genes, we have determined expression profiles of serum-derived small EVs in six samples of CRC patients (arrays 1–6) and six samples of healthy controls (arrays 7–12) using Clariom D Pico Assays as this technology should be able to analyze more than 540,000 transcripts from

Table 2: Assessment of efficiency of RNA extraction kits using RT-qPCR analyses of selected protein-coding genes and long non-coding RNAs.

Sample	18S [$C_T \pm SD$]	GAPDH [$C_T \pm SD$]	B2M [$C_T \pm SD$]	MALAT1 [$C_T \pm SD$]	ZFAS1 [$C_T \pm SD$]	GAS5 [$C_T \pm SD$]
MagMAX 1[a]	19.13 ± 0.10	27.83 ± 0.04	19.19 ± 0.25	26.66 ± 0.02	24.01 ± 0.04	ND
MagMAX 2[a]	18.72 ± 0.07	28.18 ± 0.07	19.26 ± 0.25	26.59 ± 0.13	24.57 ± 0.08	ND
MagMAX 3[a]	18.11 ± 0.03	27.69 ± 0.05	18.14 ± 0.07	25.17 ± 0.02	23.83 ± 0.09	ND
Monarch 1[b]	19.38 ± 0.07	27.54 ± 0.15	20.28 ± 0.07	26.47 ± 0.08	25.80 ± 0.08	25.92 ± 0.25
Monarch 2[b]	19.22 ± 0.04	28.17 ± 0.16	19.92 ± 0.08	27.10 ± 0.17	25.80 ± 0.15	ND
Monarch 3[b]	18.46 ± 0.19	25.01 ± 0.05	19.59 ± 0.00	24.81 ± 0.04	26.77 ± 0.09	27.91 ± 0.10
Norgen Biotek 1[c]	22.03 ± 0.18	29.87 ± 0.01	21.20 ± 0.11	26.48 ± 0.07	25.49 ± 0.00	ND
Norgen Biotek 2[c]	23.05 ± 0.19	30.35 ± 0.25	21.27 ± 0.25	28.46 ± 0.14	28.87 ± 0.12	ND
Norgen Biotek 3[c]	23.86 ± 0.16	32.79 ± 0.25	24.32 ± 0.25	30.17 ± 0.25	29.43 ± 0.25	ND

C_T, treshold cycle; SD, standard deviation; B2M, beta-2-microglobulin; ND, non-detected. [a]MagMAX – MagMAX™ *mir*Vana™ Total RNA Isolation Kit (Applied Biosystems, Waltham, MA, USA), [b]Monarch – Monarch® Total RNA Miniprep Kit (New England BioLabs, Ipswich, MA, USA), [c]Norgen Biotek – Exosomal RNA Isolation Kit (Norgen Biotek, Thorold, Canada).

as little as 100 pg of total RNA. In all cases, a yield of cRNA was higher than 62 µg after 12 cycles of PCR, which is in accordance with kit expectations. Further, 20 µg of cRNA were used for subsequent synthesis of ds-cDNA, which should result in yields higher than 6.6 µg. This step was also successfully completed as all samples provided yields higher than 12 µg of ds-cDNA. Additionally, the efficiency of hybridization was verified using hybridization controls added to the samples during the procedure and all samples passed successfully. Moreover, quality control from probe level model was performed using relative log expression (RLE) and normalized unscaled standard error (NUSE) metrics to assess the quality of individual arrays. As expected, RLE values were between −0.06 and 0.04, while NUSE values were between 1.00 and 1.02 indicating the similar quality of analyzed chips (Figure 3A and B). Finally, quality control of raw data was performed using "arrayQualityMetrics" to get feedback on the whole experimental procedure and identify potential outliers. Totally, three different approaches were used including between array comparison (Figure 4A and B), array intensity distribution (Figure 4C and D), and individual array quality (Figure 4E and F). Based on the results of Kolmogorov–Smirnov test, two samples of healthy controls (arrays 8 and 9) were considered to be outliers as their K_a values were higher than determined treshold (Figure 4C). Nevertheless, the other used methods did not mark these samples as outliers. As the outlier detection is a poorly defined question and performed methods serve only as hints, which are intended to be followed up manually, we decided not to exclude these two arrays from further analyses.

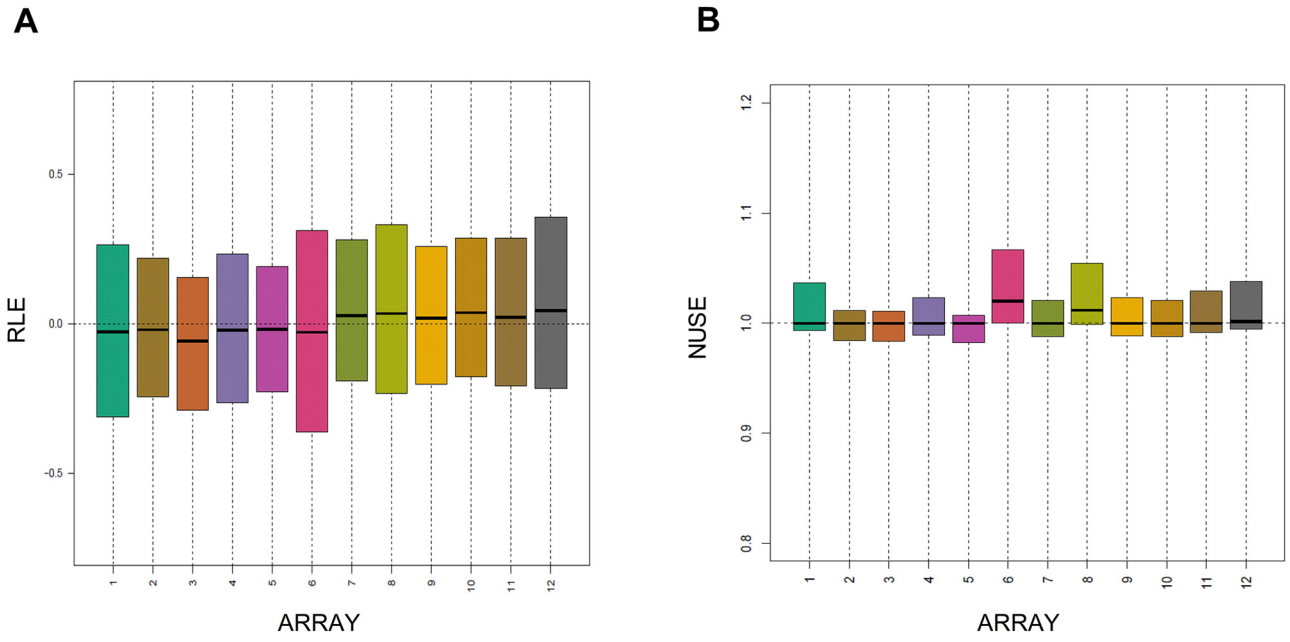

Figure 3: Quality control of samples analyzed by Clariom D Pico Assays from probe level model. (A) Quality control from probe level model using relative log expression (RLE) metric (expected values should be around 0). (B) Quality control from probe level model using normalized unscaled standard error (NUSE) metric (expected values should be around 1). Arrays 1–6 = colorectal cancer patients, arrays 7–12 = healthy controls.

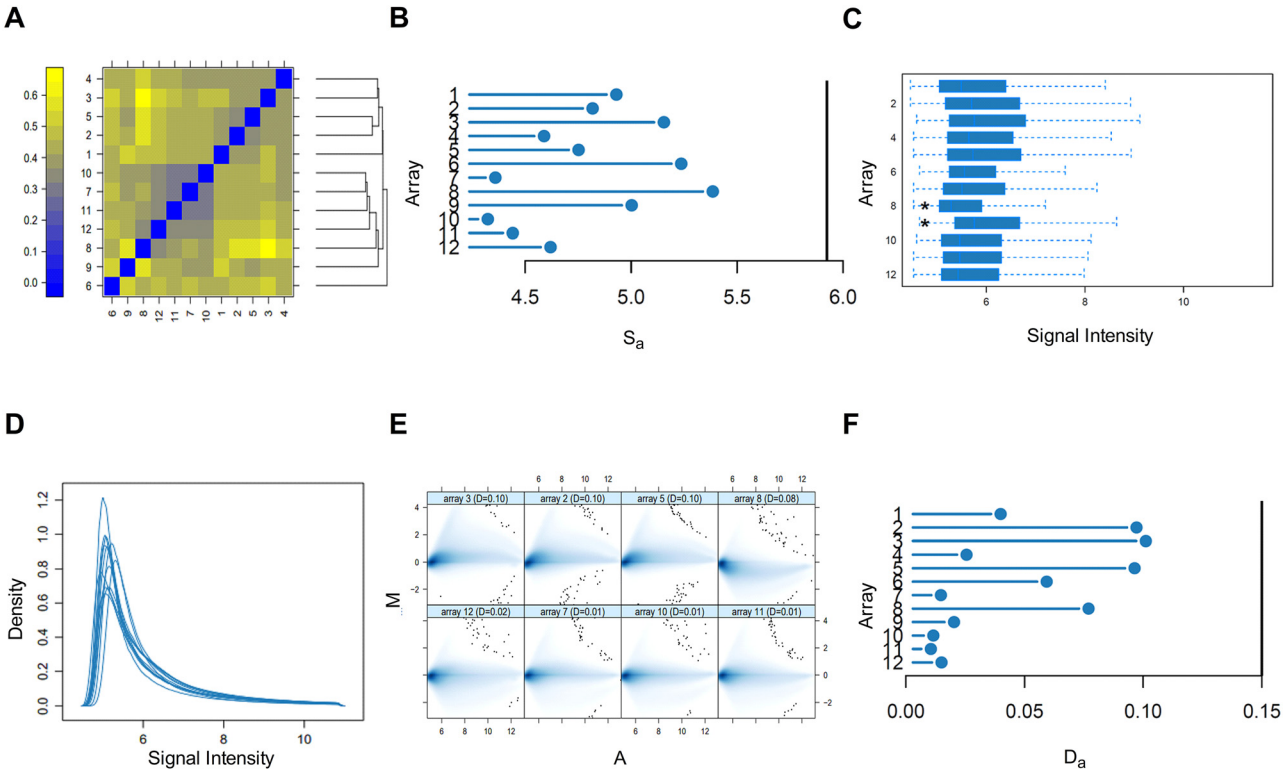

Figure 4: Quality control of samples analyzed by Clariom D Pico Assays using "arrayQualityMetrics". (A) A false color heatmap of the distances between arrays. (B) A bar chart of the sum of distances to other arrays (a treshold of 5.92 was determined indicating the outlier samples). (C) Boxplots representing summaries of the signal intensity distributions of analyzed arrays (outlier detection performed by computing the Kolmogorov–Smirnov statistic K_a between each array's distribution and the distribution of the pooled data). (D) Density estimates (smoothed histograms) of the data. (E) MA plots (four arrays with the highest and lowest values of Hoeffding's statistic Da are shown). (F) A bar chart of the Da (a treshold of 0.15 was determined indicating the outlier samples). Arrays 1–6 = colorectal cancer patients, arrays 7–12 = healthy controls, *samples indicated as outliers.

Comparing CRC samples and healthy controls, 7,182 transcripts were identified to be significantly deregulated between these two groups of samples (adjusted p<0.05). The majority of them were non-coding RNAs with more than 70 % being lncRNAs, while protein-coding genes represented the second most common gene biotype (Table 3). The hierarchical clustering showed that CRC samples may be distinguished from healthy controls based on the different expression of all significant genes (Figure 5A), protein-coding genes (Figure 5B), or lncRNAs separately (Figure 5C) (adjusted p<0.05). Altogether, our results confirm the suitability of Clariom D Pico Assays for expression profiling of small EVs isolated from low volume of sera.

Discussion

EVs have been intensively studied over the past decade, especially due to their abundance in different body fluids as well as their ability to transport various biomolecules. Thus, they could serve as novel promising targets of translational cancer research. However, despite a huge efforts, their clinical application is still highly restrained due to the lack of standardized methods for EVs isolation and characterization as well as non-existence of validated protocols for high-throughput screening of novel biomarkers. The major obstacles are the complexity of samples, overlapping biochemical and physiochemical characteristics between different EVs subtypes, the diversity of vesicles, and currently limited understanding of their biogenesis and release. Therefore, numerous isolation techniques based on different principles introduce significant variations in the concentration, size, and purity of gained EVs [38–40].

In the present study, we have compared a performance of three different methodical approaches commonly used for the isolation of small EVs from human blood serum to get the purest fraction of small EVs in sufficient quantity. As the volume of human samples in clinical settings is often limited, we decided to optimize the isolation procedure using only 150 µL of serum. Based on the results of TEM and DLS, we can conclude that EVs isolated by kits from Norgen Biotek and Exiqon were characterized by high PDI values indicating the

Table 3: An overview of the significantly deregulated gene biotypes in serum-derived small extracellular vesicles of colorectal cancer patients compared to healthy controls analyzed by Clariom D Pico Assays (adjusted p<0.05).

Gene biotype	Class of RNA	Total number of transcripts	Percentage from all identified genes, %	Percentage from particular gene biotype, %
Non-coding RNAs		**3,137**	**44.0 %**	–
	lncRNAs	2,309	32.0 %	74.0 %
	miscRNA	304	4.5 %	10.0 %
	miRNA	269	4.0 %	9.0 %
	snRNA	166	2.5 %	5.0 %
	snoRNA	80	1.0 %	2.0 %
	scaRNA	3	0.0 %	0.0 %
	rRNA	3	0.0 %	0.0 %
	ribozymes	2	0.0 %	0.0 %
	mt_tRNA	1	0.0 %	0.0 %
Protein-coding		**2,659**	**37.0 %**	–
Pseudogenes		**1,285**	**18.0 %**	–
	Processed	845	12.0 %	66.0 %
	Unprocessed	352	5.0 %	27.0 %
	rRNA	52	1.0 %	4.0 %
	Unitary	33	0.0 %	3.0 %
	Polymorphic	3	0.0 %	0.0 %
Others		**101**	**1.0 %**	–

lncRNA, long non-coding RNA; miscRNA, miscellaneous RNA; miRNA, microRNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; scaRNA, small cajal body-specific RNA; rRNA, ribosomal RNA; mt_tRNA, mitochondrial transfer RNA.

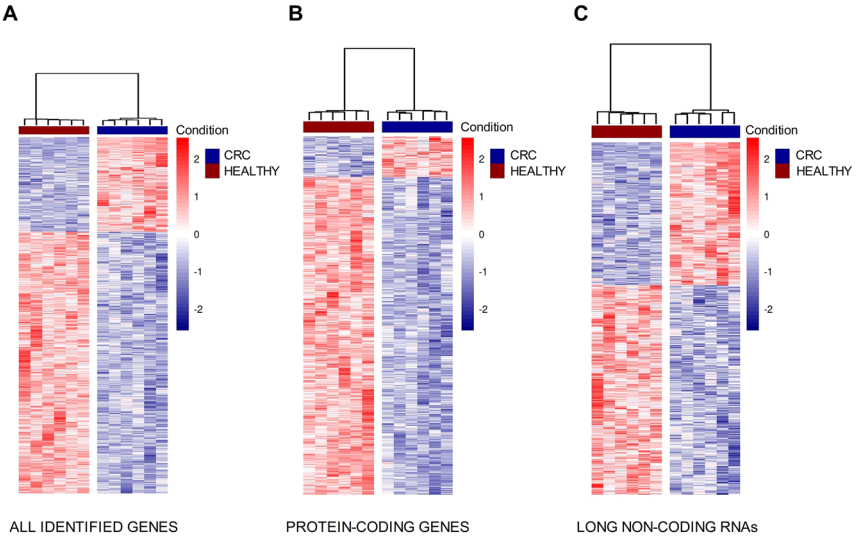

Figure 5: The hierarchical clustering of six colorectal cancer (CRC) samples and six healthy controls based on the significantly different expression (adjusted p<0.05) of (A) all identified genes, (B) protein-coding genes, (C) long non-coding RNAs.

presence of particles with very broad size range and all samples contained a considerable number of smaller particles. In case of SEC, F6 and F7 were proved to contain mostly the particles with the expected size of small EVs, although the detectable amount of ApoB was also confirmed by WB. These data confirm the suitability of qEVsingle/35 nm columns for small EVs isolation from low volume of sera as this approach enable to get high number of vesicles with proper size, shape, and sufficient purity although the lipoproteins are co-isolated to some extent.

Extraction of RNA is a crucial step for gene expression studies. As we were working with a low amount of starting material, we struggled for maximum yields. Previously, Eldh et al. [15] compared seven different approaches for the

extraction of RNA from EVs including kits from four companies specialized in RNA extractions. Interestingly, spin column-based methods showed higher yields compared to phenol-based methods including combined phenol- and column-based techniques. This could be caused by a more rigid membrane of EVs enriched for cholesterol and sphingomyelin leading to incomplete lysis by phenol. Further, Bioanalyzer electrophoretograms of RNA patterns proved a significant difference in the relative presence of small and long RNAs depending on the isolation method. While column-based techniques isolated RNA with a broad size distribution, methods involving phenol were confirmed to be more efficient for isolation of small RNAs. Thus, to find the best approach for the extraction of RNA from our small EVs, we compared three different commercial kits not involving phenol. We planned to compare the kits' effectiveness by RNA concentration measurements, however, it was too low to be reliably quantified. Thus, we assessed the efficiency by RT-qPCR analyses of selected genes as well as by comparing the results from two independent samples analyzed directly by selected microarrays. The best results were obtained in case of samples isolated by Monarch Total RNA Miniprep Kit, thus we decided to use this approach for all samples designed to transcriptome profiling.

The suitability of isolated RNA for high-throughput screening of lncRNAs as well as protein-coding genes was assessed using six samples from CRC patients and six samples from healthy controls. So far, several different studies investigated the expression profiles of small EVs using microarray technology [41–43]. Nevertheless, no one has tested the appropriateness of Clariom D Pico Assays for transcriptome profiling of serum-derived small EVs in CRC so far. Despite the low concentration of isolated RNA, we gained high yields of ds-cDNA and all samples passed the quality as well as hybridization control. The annotation of specific genes emerged to be rather complicated task due to the lack of updates in original annotation files and the use of probes derived from outdated transcripts that are no longer present in current databases. Thus, we were forced to create our own CDFs using probe sequences from BRAINARRAY project and GENCODE database as a target. Differential expression analysis revealed more than 7,000 transcripts significantly deregulated between compared groups including a high portion of lncRNAs and protein-coding genes. Importantly, further analyses confirmed our initial hypothesis that expression profiles of small EVs are significantly different between CRC patients and healthy controls, thus selected RNA molecules could serve as promising non-invasive diagnostic biomarkers in the future.

To summarize, we have achieved our objective of protocol optimization. Importantly, our results confirm the suitability of Clariom D Pico Assays for transcriptome profiling of small EVs isolated from low volume of sera indicating the possible utilization in clinical settings. Nevertheless, the use of expansion cohorts is necessary and robust validation by independent method should be performed. In addition, there is an increasing evidence that EVs are highly heterogenous consisting of several subpopulations with its own biological role and wide spectrum of effects on recipient cells [44–47]. Thus, it is crucial to develop novel methods enabling precise separation of particular subpopulations as well as to identify reliable markers characteristic for distinct EVs subtypes. Finally, studies focusing on EVs isolation directly from cancer tissues are highly desirable [48, 49] in order to better understand the role of these vesicles in the tumor microenvironment and to perform comprehensive molecular characterization of disease-related EVs.

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Research ethics: The study was approved by the local Ethics Board at Masaryk Memorial Cancer Institute (ID 2018/1671/MOU) and conforms to recognized standards of Helsinki Declaration.

Informed consent: Informed consent was obtained from all individuals included in this study.

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