Application Note

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Exosomal miRNA Analysis with Agilent Bioanalyzer and SurePrint miRNA Microarray

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Abstract

Exosomal microRNAs (miRNAs) are a unique type of RNA that is important in cell-cell communication and is protected from RNase degradation due to being enclosed in the exosome. Recently, exosomal miRNAs have gained interest as a minimally invasive biomarker for liquid biopsies. However, these miRNAs can be difficult to detect and analyze due in part to their low abundance.

In this study, we tested the ability of various kits to extract RNA from 1 mL or 3.7 mL of serum with exosomal enrichment, then performed quality control (QC) with the 2100 Bioanalyzer and analyzed miRNAs with the SurePrint G3 Human miRNA microarray. Our data indicates exosomal RNA is detectable by the Bioanalyzer as a smear peak. Additionally, we analyzed differentially expressed miRNAs extracted from 2 mL of serum from cancer patients and normal controls using the SurePrint G3 Human miRNA microarray. This study indicates the Agilent miRNA microarray are a powerful tool to detect and analyze miRNAs from liquid biopsy without modifying the standard microarray procedure.

Introduction

Exosomes are small vesicles that have important roles in cell-cell communication and, in recent years, have been used as analytes in liquid biopsies. Exosomes also transport RNAs (including miRNAs), where they are protected from degradation by RNases. While the function of exosomes and exosomal RNAs are still unclear, it has been reported that exosomal miRNAs released from cancerous cells can relate to tumorigenesis, metastases, and induce acquisition of drug tolerance in other cells[1]. Due to these factors, exosomal miRNAs are of interest as potential as non-invasive biomarkers, but they have proven difficult to analyze due in part to their extremely low concentration, which can fall beneath the detection threshold of conventional spectrophotometers[2].

Agilent provides a full workflow for the analysis of miRNA, starting from sample QC up to final analysis. This workflow includes the Agilent 2100 Bioanalyzer, the SurePrint miRNA microarray platform, and the GeneSpring GX software. The Agilent 2100 Bioanalyzer is an electrophoresis instrument that enables the analysis DNA and RNA at a wide variety of concentrations using microfluidic chips. The Agilent miRNA microarray platform is a useful tool for the direct detection of miRNAs from tissues, cells, and FFPE samples without reverse transcription and amplification. In this study, we examined several commercially available kits for the extraction of exosomal RNA from serum and evaluated these samples with the Bioanalyzer RNA 6000 Pico assay. Additionally, we analyzed these samples with the SurePrint miRNA microarray to identify differentially expressed miRNAs between cancer patients and normal controls.

Experimental

Exosomal RNAs were extracted from commercially available serum (PromedDx) by DNA Chip Research Inc. Serum samples were stored at –80 °C; after thawing, samples were filtered using a 0.22 µm filter to remove contaminants.

We evaluated exosomal RNA extracted from three normal samples (A, B, and C; female, caucasian, and aged in their 70s) using multiple commercially available kits. The extraction kits and volume of serums for evaluation are summarized in Table 1. To test the number of detected miRNAs by microarray, normal sample A was extracted from either 1 or 2 mL of serum using the exoRNeasy Maxi Kit (Qiagen). To analyze differentially expressed miRNAs, 2 mL of 6 serum samples (normal samples A, B, and C; cancer patient samples D, E, and F) were used. Extractions were performed according to manufacturer's instructions.

All extracted RNA samples were subjected to electrophoresis on the Agilent Bioanalyzer using the RNA 6000 Pico assay (Agilent p/n 5067-1513). Universal Human miRNA Reference RNA (Agilent p/n 750700, referred to as miR UniRef in this note) was also used as a positive control for the electrophoresis to allow the evaluation of the possible effects of salt or other contaminants in sample buffer, that may affect the electropherogram generated from the Pico assay. Following extraction, RNA samples were concentrated by speed vacuuming and diluted to 9 ul using nuclease-free water (Thermo Fisher Scientific, p/n 10977015).

We labeled 2 µl of concentrated RNA samples and hybridized to the SurePrint G3 Human miRNA microarray 8x60K Rel. 21.0 following the manufacture's protocol (Agilent miRNA microarray protocol version 3.1.1, G4170-90011). Since it was difficult to measure RNA quantity because of low concentration, we normalized sample inputs by volume (2 µL) rather than total RNA quantity.

Labeling Spike-In (part of the miRNA Spike In kit, Agilent p/n 5190-1934) was added to each RNA sample, which was then labeled with Cy3-pCp using the miRNA Complete Labeling and Hyb kit (Agilent p/n 5190-0456). Labeled samples were purified with MicroBio Spin 6 columns (BioRad p/n 732-6221). Purified samples were hybridized to miRNA microarray slides with Hyb Spike-In (part of the miRNA Spike In kit p/n 5190-1934) at 55 °C for 20 hours.

As an experimental positive control, 100 ng of miR UniRef was labeled and hybridized to two arrays for every slide. Following hybridization, the array slides were washed according to the standard protocol and scanned using the Agilent SureScan DNA microarray system. Scanned data were analyzed using Agilent Feature Extraction and Agilent GeneSpring GX software.

As the purpose was to evaluate the difference of starting amounts of serum, no normalization between arrays was performed. We note that a 90th percentile shift is recommended to analyze miRNA array data for tissue and cell samples.

Results and Discussion

The electropherograms from the Bioanalyzer showed smeared peaks, with an overall trend towards detecting higher peaks when greater serum volumes were used for extractions (Figure 1).

Some exosomal RNA extracted using two different Qiagen kits demonstrated multiple peaks not only in the small RNA region, but also in higher molecular weight regions. Repeated electrophoretic analyses with 3-fold diluted samples gave similar results (data not shown). As the resulting ladder, miRNA UniRef, and blank lane traces were normal (Figure 1a and b), these runs were still included. RNA samples extracted using the Total Exosome Isolation from Serum and Total Exosome RNA & Protein Isolation kits (Thermo Fisher Scientific, part numbers 4478360 and 4478545) showed a smear peak in the small RNA region. However, while sample B exhibited sufficient noise to render the RNA peak undetectable, we judged the electrophoresis successful due to the presence of normal data outside of the exosomal RNA lanes.

Table 1. Summary for extraction kits and serum volumes.

Extraction Kit	Number of Extraction	Serum Volume for Extraction (mL)	Final Elution Volume from Column (µL)	Elute Volume (µL)
exoRNeasy Serum/Plasma Midi Kit (Qiagen p/n77044)	Normal A, B and C, each n=2	1	14	10~12.8
exoRNeasy Serum/Plasma Maxi Kit (Qiagen p/n 77064)	Normal A, B and C, each n=2	3.7	14	11.8~12.6
Total Exosome isolation from serum and Total Exosome RNA & Protein Isolation Kit (Thermo Fisher Scientific p/n 4478360 and 4478545, Total Exosome Kit in this note)	Normal A, B and C, each n=2	1	100	85.6~100

All 18 samples were analyzed following a standard miRNA microarray protocol. As expected, the entire signal intensity of arrays for exosomal RNA samples were very low comparing to miR UniRef data. The Agilent Feature Extraction software provides several metrics for each array to help evaluate the quality of the array data. CV% represents the coefficient of variation of signal intensity between replicated probes. A lower signal intensity tends to correlate with a higher CV%, indicating lower reproducibility in a given array. In this study, the CV% for exosomal RNA samples were slightly higher than miR UniRef (however, they did not exceed the ideal value of 15% (Figure 2)).

The Feature Extraction software includes an automated and validated analysis workflow to determine whether the signal intensity of each miRNA is detected above noise during the extraction of signal intensity from scanned microarray images. Due to the expected low signal intensity, since the amount of RNA in exosomal sample is limited we expect it to generate low signal intensity. For this reason one of the parameter evaluated was the number of miRNA called as detected (Figure 3). As expected, the number of detected miRNAs was lower with lower starting volumes and increased proportionally when higher volumes of serum were used. However it is important to highlight that extraction kit used and starting serum volume are introducing some variability in the results, mostly focused within low expressed miRNA. This bias is most likely linked to small changes introduced by the protocol and linked to the intrinsic nature of those small molecules. It is therefore important to start with the same volume of serum and use the same RNA extraction kit within a project, in order to normalize data and limit experimental variability.

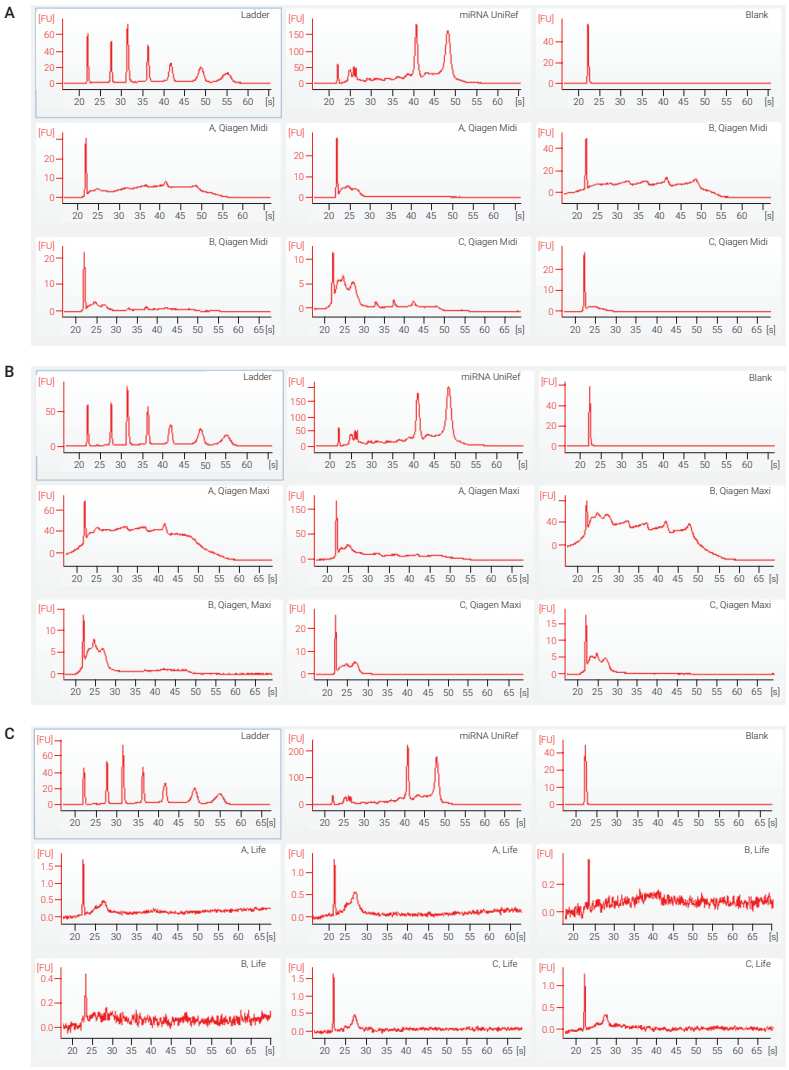

Figure 1. Electropherogram of exosomal RNA measured by Bioanalyzer RNA 6000 pico assay. *All data include Ladder, miRNA UniRef and blank lane.*

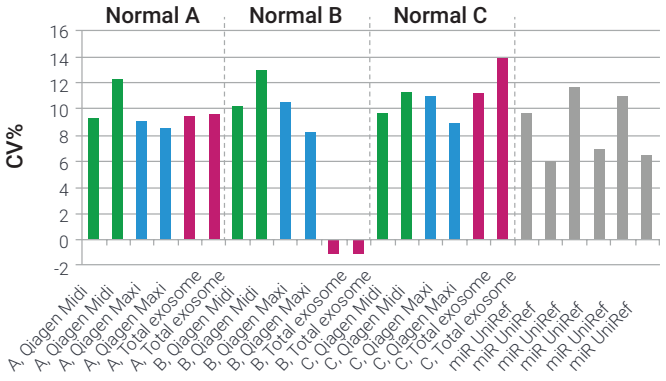

Figure 2. CV% of replicated probes in each array.

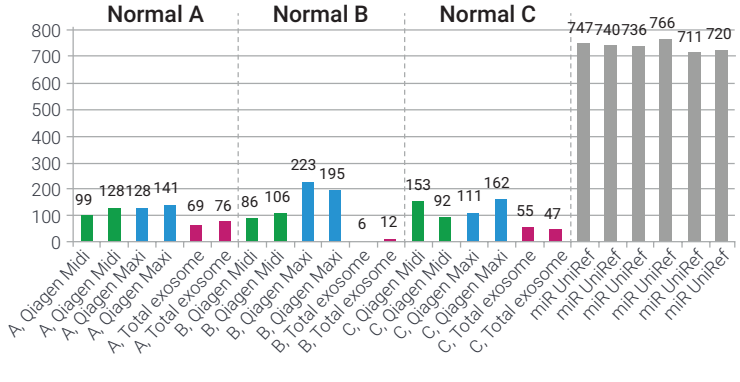

Figure 3. Number of miRNAs called as 'Detected'.

Figure 4 shows the correlation value (calculated by GeneSpring GX software) between 6 samples extracted from 3.7 ml of serum using the exoRNA Maxi Kit. As expected, the reproducibility between two datapoints from same sample was higher than data derived from different samples. This would enable the detection of differential expression, as well as indicating the differential electropherogram from the Bioanalyzer would not affect the microarray data. This is likely due to the fact the Agilent miRNA microarrays directly label miRNA and are designed to detect only mature miRNAs. This limits bias introduced into the sample compared to other technologies requiring multiple modification steps in order to detect such small molecules.

We next investigated what volume of serum is needed to analyze miRNA expression with the Agilent microarray platform. Serum sample A was divided into three tubes of 1 mL each and three tubes of 2 mL each (for a total of six tubes), then RNA was extracted using the exoRNeasy Maxi kit. Smear peaks similar to those in Figure 1A and B were detected by the Bioanalyzer system (data not shown). miRNAs were reliably detected in both 1 and 2 mL of serum (Figure 5). We opted to use 2 mL of serum due to it being a realistic volume, as well as the fact that more miRNAs may be detectable when greater volumes are used and/or when eluted samples are concentrated to 2 µL for labeling reactions.

Analysis of differentially expressed miRNAs

We analyzed differentially expressed miRNAs between 3 normal (A, B and C) and 3 lung cancer (D, E and F) patient samples. 2 mL of serum was used for each sample. To evaluate the potential impact of the starting volume of cancer samples, 1 mL of sample F was also analyzed as a separate sample (sample G).

More miRNAs were detected on cancer samples compared to normal samples (Figure 6). The scatter plots between biological triplicates were tight indicating high reproducibility within each group (Figure 7). Additionally, the scatter plot between the array data of samples F and G reflects the effect of different serum starting amounts with cancer patients, as sample G was plotted on a ½ line (Figure 8).

Generally speaking, a normalization between array data that is adopted prior to biological analyses can help limit experimental error. However, no control (e.g., 'housekeeping') exosomal miRNAs have been reported for normalizing samples. While three miRNAs were previously used for inter-sample normalization for the analysis of serum miRNAs (albeit without exosomal enrichment)[3], the profiles of these

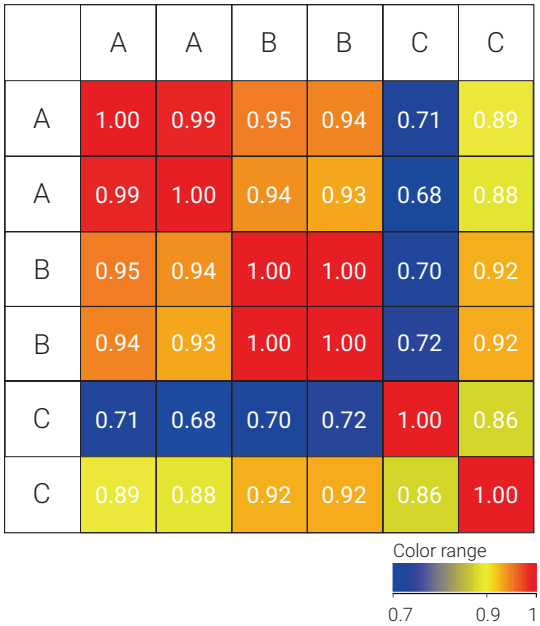

Figure 4. Correlation value between samples extracted using exoRNAeasy Maxi Kit from 3.7 mL of serum (75 miRNAs detected in all 6 data).

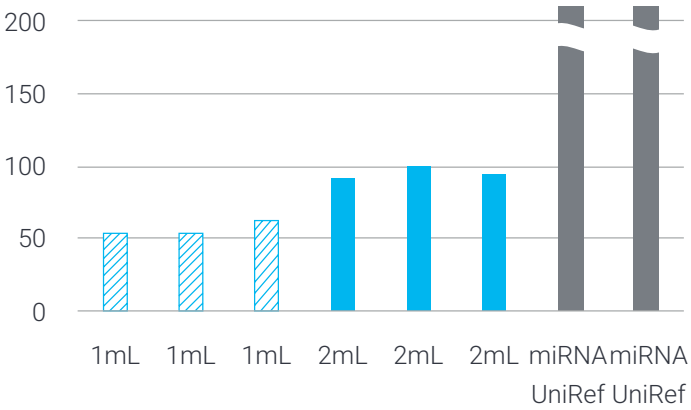

Figure 5. Number of detected miRNAs from 1 mL and 2 mL of serum using exoRNAeasy Maxi Kit.

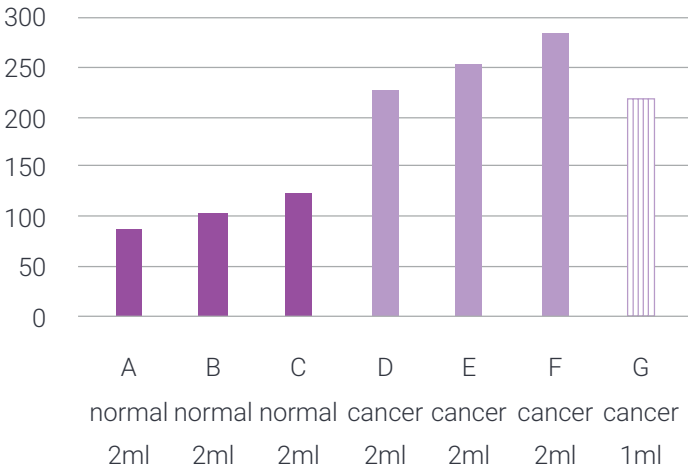

Figure 6. Number of detected miRNAs from normal and cancer samples.

Figure 7. Scatter plots between six arrays. Red rectangles indicate biological triplicates.

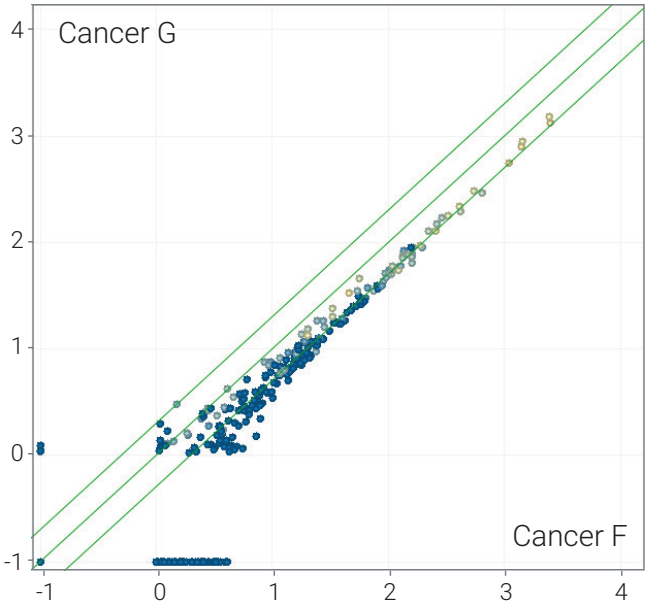

Figure 8. Scatter plot of F (2 mL) and G (1 mL) from same cancer sample. Log10 signal intensity without normalization. Green line: the center and 2-Fold.

3 miRNAs were either not detected or not stable between groups in this study. We opted to analyze data without normalization since differences in the starting amount of cancer serum was reflected in the array data and we normalized the starting serum amount to 2 mL. Additionally, as it has been reported that more exosomes are released from cancerous tissue than normal tissues[4], the difference of detected miRNA number between normal and cancer samples would likely not be attributable to experimental error.

Differentially expressed miRNAs between normal and cancer samples were analyzed using GeneSpring GX software. After filtering miRNAs that were not detected in both normal and cancer samples, 185 miRNAs were called as differentially expressed (unpaired T-test, corrected p-value cut-off of < 0.05, fold change > 2.0). Only miR-150-5p was down regulated, which targets cancer related genes like MYB and MDM4. Some upregulated miRNAs, such as miR-6794-5p and miR-4467, were previously reported as upregulated in lung cancer[5]. Other upregulated miRNAs—specifically, miR-3195, miR-4459 and miR-1273g-3p—were previously reported as biomarkers of ovarian cancer, along with other miRNAs[6]. The 52 miRNAs out of 184 miRNAs which were differentially expressed and detected in both normal and cancer are listed in Table 2.

Targeted transcripts of the 184 upregulated miRNAs were predicted using the target prediction function of the GeneSpring GX software. Next, a gene ontology analysis was performed for the 2,073 transcripts predicted to be targeted by these miRNAs. In addition to various developmental systems, some terms related to the brain—such as 'neuron' and 'synapse'—were called (Table 3).

Conclusion

Recently, small RNA-Seq has been used to analyze miRNAs; however, this method has several challenges, including complicated library preparation steps and the detection of multiple non-miRNA small RNA species[7]. The Agilent miRNA microarray platform offers a simple and powerful tool to analyze known mature miRNAs. It offers a unique workflow that includes direct labeling of total RNA samples directly from tissues and cells, limiting bias introduced to the sample. This study shows that the Agilent SurePrint G3 miRNA microarray was able to detect exosomal miRNAs from serum and analyze differentially expressed miRNAs, which may be useful for analyzing exosomes from other liquid biopsy analysis (such as plasma).

Table 2. Differentially expressed 52 miRNAs

hsa-miR-1202	hsa-miR-4459	hsa-miR-5787	hsa-miR-6800-5p
hsa-miR-1207-5p	hsa-miR-4466	hsa-miR-6087	hsa-miR-6819-3p
hsa-miR-1225-5p	hsa-miR-4516	hsa-miR-6088	hsa-miR-6821-5p
hsa-miR-1273g-3p	hsa-miR-4532	hsa-miR-6089	hsa-miR-6850-5p
hsa-miR-1908-3p	hsa-miR-4634	hsa-miR-6090	hsa-miR-6869-5p
hsa-miR-2861	hsa-miR-4687-3p	hsa-miR-6124	hsa-miR-6879-5p
hsa-miR-3195	hsa-miR-4767	hsa-miR-6125	hsa-miR-6891-5p
hsa-miR-328-5p	hsa-miR-4778-5p	hsa-miR-6165	hsa-miR-7107-5p
hsa-miR-3610	hsa-miR-4787-3p	hsa-miR-630	hsa-miR-7110-5p
hsa-miR-3663-3p	hsa-miR-483-5p	hsa-miR-638	hsa-miR-7150
hsa-miR-3665	hsa-miR-5100	hsa-miR-642a-3p	hsa-miR-762
hsa-miR-3960	hsa-miR-5703	hsa-miR-6510-5p	hsa-miR-765
hsa-miR-4281	hsa-miR-5739	hsa-miR-6740-5p	hsa-miR-8069

Table 3. GeneOntology results (Top 15 terms)

GO ACCESSION	GO Term	p-value	corrected p-value
GO:0048731	system development	1.25538E-30	1.51032E-25
GO:0007399	nervous system development	6.07322E-29	3.65326E-24
GO:0007275	multicellular organism development	1.42849E-27	5.72861E-23
GO:0048856	anatomical structure development	1.48083E-26	4.45387E-22
GO:0032502|GO:0044767	developmental process	2.77794E-25	6.68414E-21
GO:0097458	neuron part	5.57105E-23	1.11706E-18
GO:0045202	synapse	1.24013E-22	2.13138E-18
GO:0044456	synapse part	2.40977E-21	3.62392E-17
GO:0030054	cell junction	3.43148E-21	4.58702E-17
GO:0005515|GO:0001948|GO:0045308	protein binding	5.26826E-21	6.33811E-17
GO:0023051	regulation of signaling	2.6237E-20	2.86955E-16
GO:0003674|GO:0005554	molecular_function	1.69804E-19	1.70239E-15
GO:0005488	binding	2.1747E-19	2.01256E-15
GO:0048522|GO:0051242	positive regulation of cellular process	1.77038E-18	1.52135E-14
GO:0022008	neurogenesis	3.87768E-18	2.91571E-14

References

1. Takahashi RU. et al., The role of extracellular vesicle microRNAs in cancer biology. *Clin Chem Lab Med*. **2017**, 55(5):648-656.
2. Garcia-Elias A. et al., Defining quantification methods and optimizing protocols for microarray hybridization of circulating microRNAs. *Sci Rep*. **2017**, 7(1):7725.
3. Bhome R. et al., Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. *Cancer Lett*. **2018** , 420:228-235.
4. Shimomura A. et al., Novel combination of serum microRNA for detecting breast cancer in the early stage. *Cancer Sci*. **2016**, 107(3):326-34.
5. Dejima H. et al., Exosomal microRNA in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer. *Oncol Lett*. **2017**, 13(3):1256-1263.
6. Yokoi A. et al., Integrated extracellular microRNA profiling for ovarian cancer screening. *Nat Commun*. **2018**, 9(1):4319.
7. Buschmann D. et al., Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. *J Extracell Vesicles*. **2018**, 7(1):1481321.

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