

Exosome miRNA Profiling with the Agilent miRNA Microarray Platform

Authors

Simona Coco¹ and Irene Vanni^{1,2},

¹Lung Cancer Unit, IRCCS Ospedale Policlinico San Martino, Genova, Italy

²Department of Internal Medicine and Medical Specialties (DIMI), University of Genova, IRCCS Ospedale Policlinico San Martino, Genoa, Italy

Andrea Carenzo,
Laboratory of Cancer Genomics, Fondazione Edo ed Elvo Tempia, Biella, Italy

Francesco L. Savasta and Ibrahim Kisakesen, Agilent Technologies, Inc., Amstelveen, The Netherlands

Abstract

Exosomes are cell-derived vesicles detected in almost all bodily fluids and they are involved in the intercellular connection by transferring biological information (e.g. DNA, RNA, and proteins). Research in the past decade point to the possible role these vesicles may play in both physiological and pathological processes. Profiling exosomal miRNAs becomes very intriguing, especially with the aim of investigating whether these molecules can be used as biomarkers. Investigators measuring exosomal miRNA concentrations often face low yields and poor quality total RNA. These issues create challenges for measuring miRNA concentrations using tools such as microarray platforms and other assays. In this Application Note, we present practical solutions that minimize the risk of sample loss when using the Agilent miRNA microarray platform, resulting in accurate exosomal miRNA measurements.

Introduction

Exosomes are cell derived nanovesicles (40 to 100 nm) found in almost all body fluids, such as plasma and serum. Exosomes are present in both healthy individuals and patients with malignancies. Increasing evidence has shown that exosomes are able to transfer molecules such as DNA, RNA (including miRNA fraction), and protein from one cell to another. In this way, they behave as “shuttles” transferring biochemical and molecular information. Due to these characteristics, exosomes and their content, such as miRNAs, play a key role in a multitude of physiological and pathological processes, including cancer, suggesting their potential application as biomarkers¹.

Nowadays, microarrays remain as one of the most powerful high-throughput technologies able to screen the expression profiles of thousands of miRNAs simultaneously in a single experiment^{2,3}. However, beginning a study on exosomal miRNA using a microarray platform can present a few challenges. The amount of total RNA that can be extracted from exosomes is scarce, below the optimum amount requested for miRNA microarray assays and for its proper quantification. The purity of the isolated exosomal total RNA can be poor (260/280 and 260/230 ratios <1), even when using column-based commercial purification kits.

This makes it complicated to accurately quantify samples, and it limits the methods that can be used. In addition, exosomes include different types of RNA molecules, depending on the physiological or pathological state of the cells that secrete them^{4,5}.

The variable composition of these vesicles makes these samples difficult to analyze on microarray, as this application is often optimized for cellular total RNA as input material⁶. All these aspects make it difficult to establish a reliable workflow that may ensure a satisfactory success rate for the experiments and that can safeguard the costs of a project and minimize the risk of loss of precious samples.

This study evaluates some practical approaches that can be considered to try to overcome some of the most common difficulties that may be met when working with exosomal miRNA samples on the Agilent miRNA microarray platform.

Experimental

Samples extraction and purification

Plasma samples were obtained from four healthy individuals and 120 donors with lung cancer. Exosomal total RNA samples were purified from 1 mL of plasma using the exoRNeasy Serum/Plasma Midi kit (Qiagen, Hilden, Germany) according to manufacturer instructions.

Sample quantification

The exosome total RNA quantification, including the presence of the miRNA fraction, was assessed using different technologies such as the NanoDrop 1000 and the Qubit 2.0 Fluorometer using the Qubit microRNA Assay kit (Thermo Fisher Scientific Waltham, MA, USA). An additional size quality control was performed using two microfluidic electrophoresis tools as the Agilent 2200 TapeStation system and the Agilent 2100 Bioanalyzer system with the Agilent High Sensitivity RNA ScreenTape and the Agilent small RNA kit, respectively. All systems were used according to manufacturer protocols.

Microarray experiment

Exosomal total RNA samples were processed using the Agilent miRNA complete labeling and hybridization kit (part number 5190-0456) and Agilent miRNA spike-in kit (part number 5190-1934). Samples were hybridized on SurePrint human miRNA microarrays (part number G4872A - AMADID: 070156).

The Agilent miRNA microarray system with miRNA complete labeling and hybridization kit protocol v.3.1.1 (G4170-90011) was followed with the following exceptions:

- Three different volumes (2 μ L, 6 μ L, and 10 μ L) of purified total RNA samples at unknown concentration were used as input amount, instead of the recommended 100 ng of total RNA.
- The supplement of a commercial synthetic DNA poly-A oligonucleotide 3' labeled pCp-Cy3, was added to the final hybridization mix. The 30-mer synthetic oligonucleotide (HPLC purified) was synthesized by TIB Molbiol s.r.l. (Genova, Italy). The lyophilized oligonucleotide was resuspended at a final concentration of 100 pmol/ μ L. Before use, an aliquot was taken and through serial dilutions brought to two final concentrations of 50 and 5 amol/ μ L.

Microarrays were scanned (3 microns, 20 bit) on an Agilent microarray scanner (part number G2505C) and data extracted using Agilent feature extraction v.12.0.0.7. QC metrics from feature extraction were used to evaluate the success of the labeling and hybridization. The Agilent feature extracted parameter *IsGeneDetected* (supported by at least 15 replicates probes) was used to estimate the number of discernible miRNAs.

Results and Discussion

Different quantification methods were tested to find out which could provide the most reliable and consistent measurements of the total RNA extracted from the exosomal vesicles, and for assessing the presence of the miRNA fraction. Spectrophotometry (NanoDrop) was not accurate because of the low concentration of the samples. Additionally, the presence of contaminants commonly detected in exosomal RNA resulted in poor 260/280 (<1.8) and 260/230 (<0.5) ratios, hindering accurate quantification. When we used an electrophoretic measurement system, the RNA ScreenTape assay on the 2200 TapeStation System, the results were still unsatisfactory because sample concentrations were too close to the limit of detection of the assay (500–10000 pg μ L). Besides, this assay is designed for analyzing RNA molecules from 50 to 6,000 nt; therefore, no information regarding the small RNA region could be extrapolated from the analysis. An alternative electrophoretic measurement may be obtained using the Agilent RNA 6000 Pico assay (total RNA) on the 2100 Bioanalyzer system, providing a lower limit of detection (50 to 5,000 pg/ μ L). However, a point that should be considered when using microfluidics-based electrophoretic tools to measure the exosomal total RNA profile is that it misses the ribosome 18S and 28S peaks normally seen in cellular total RNA measurements, preventing the RNA integrity assessment.

Due to the low yields and low accuracy of the spectrophotometric and electrophoretic methods, we decided to focus our attention on the miRNA fraction in the samples by using the Qubit microRNA assay kit. This proved to be a fast and reproducible quantification method. We also tried the small RNA assay by the 2100 Bioanalyzer system, aiming to profile the small RNA fraction in our samples, as described in a previous Agilent Application Note⁷. The comparison of the small RNA assay with the results obtained from the Qubit tool showed similar data, with the small RNA fraction too close to the detection limit of the small RNA Bioanalyzer assay.

Based on the difficulties measuring total RNA from exosomes, we decided not to follow the 100 ng total RNA input requirement recommended by the Agilent miRNA microarray protocol. We moved our focus on the miRNAs fraction to try to determine the minimum amount of material necessary to carry out our study. For this study, we used four plasma samples from healthy donors and tested different input volumes of the purified samples regardless of the final concentration. Therefore, 2 μ L, 6 μ L, and 10 μ L from each sample was used as input for the miRNA labeling and hybridization protocol. Another issue we encountered running miRNA microarray analyses on exosomal miRNAs was the poor signal obtained from the Bright Corners on the Agilent microarrays (Figure 1).

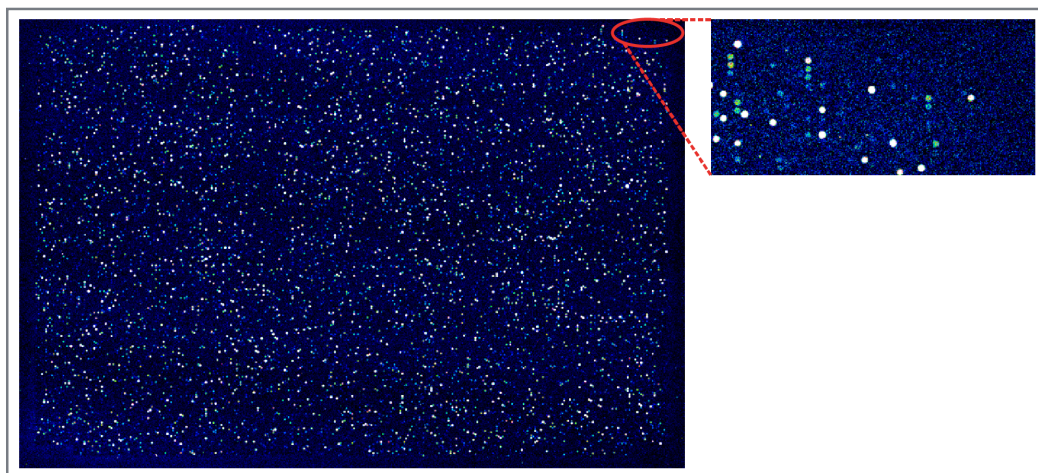


Figure 1. An example of array in which the positive control features placed at the corners (Bright Corners) cannot be identified. These positive controls are used by the analysis software to determine the position of all the other features. The success of this step during the analysis is critical for the success of the data extraction. Working with exosomal miRNAs, the Bright Corners often show very dimmed or no signal.

The feature extraction software uses these corner control features to correctly place the grid file, which is necessary for successful data extraction. Signals from these corner features were so dim that they could not be distinguished from the background noise. This issue made it impossible to use the software feature that allows manual placement of the grid (Figure 2).

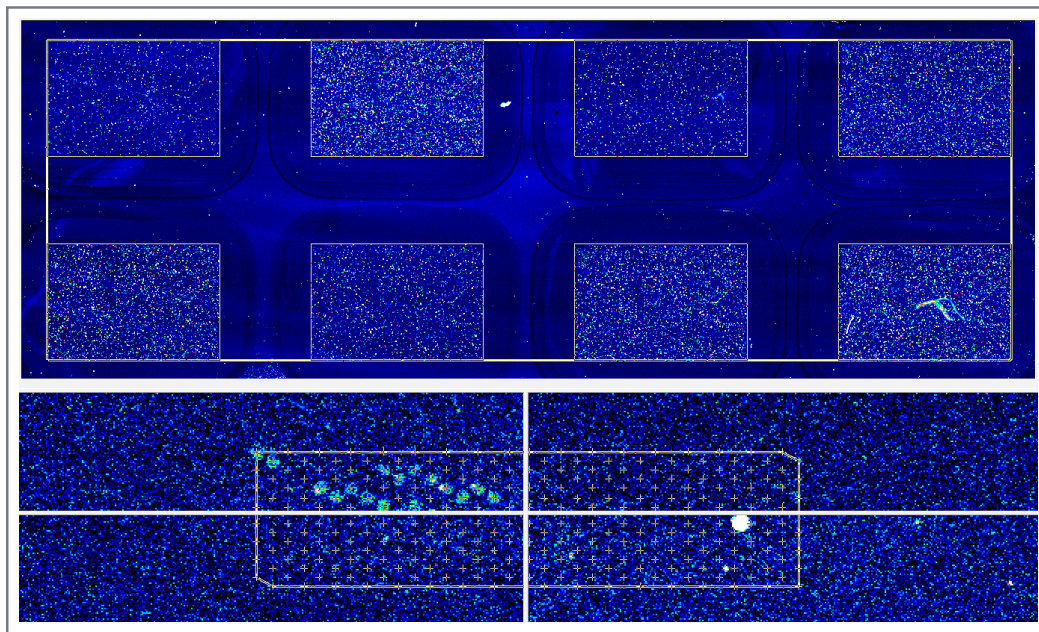


Figure 2. Manual Gridding mode in Feature Extraction: this mode can be used to manually place the grid file on the corresponding array features. The main grid is placed using as fix points the Bright Corners of the arrays at the extremities of the slide. This task proves to be impossible when signal from the Bright Corners is extremely low or at the same level of the background noise.

To overcome this problem, we implemented the use of a synthetic oligonucleotide, consisting of a DNA poly-A stretch 3' labeled with pCp-Cy3, and we planned two experimental conditions for each volume of input sample tested, adding either 5 or 50 amol of the synthetic oligonucleotide to each hybridization mix. The results indicated that the most reliable quantity of synthetic oligonucleotide to be used to obtain a clear and consistent signal from the Bright Corners is 50 amol (Figure 3).

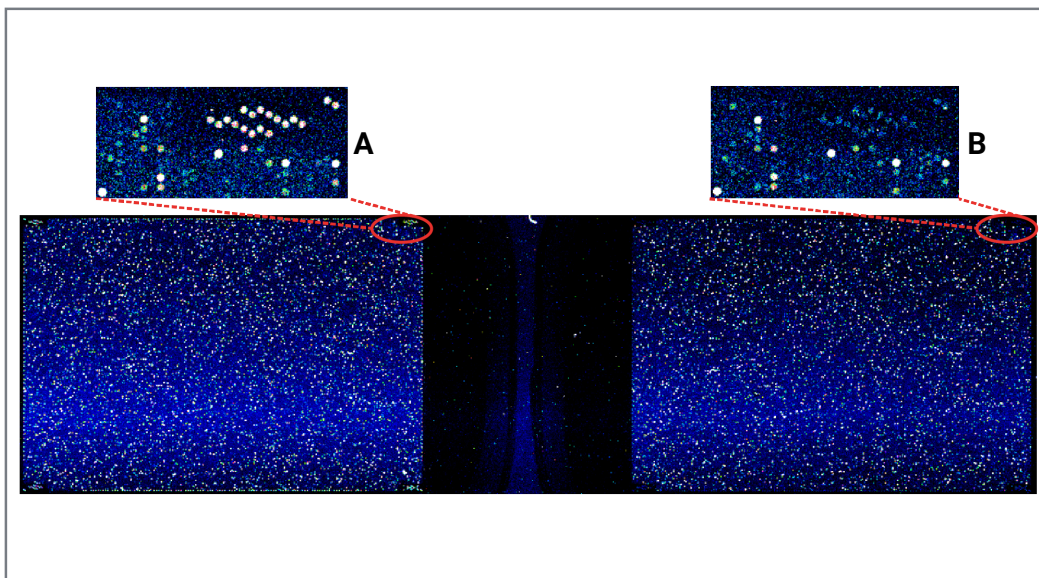


Figure 3. (A) Array hybridized spiking 50 amol of the poly-A synthetic oligo in the hybridization mix. The Bright Corners showed consistently very good signal intensity, allowing the software to easily identify these features. No gridding issues were experienced since the use of this amount of synthetic oligo was implemented in our protocol. (B) Array hybridized spiking 5 amol of the poly-A synthetic oligo in the hybridization mix. The Bright Corners showed signals still too close to background levels, causing still failure of the automatic gridding in some cases. This amount of synthetic oligo proved to be not sufficient to provide reliable and consistent success of the automatic gridding operated by the software.

When using 5 amol of the oligonucleotide, for some conditions, the automatic gridding still failed or the signal from the bright corners was still close to the background noise.

The results obtained for the four normal samples showed that all volumes (2 μ L, 6 μ L, and 10 μ L) used were sufficient for the detection of miRNAs. They also displayed consistency between the input volume and the number of miRNAs that the system could detect for these healthy samples (Figure 4).

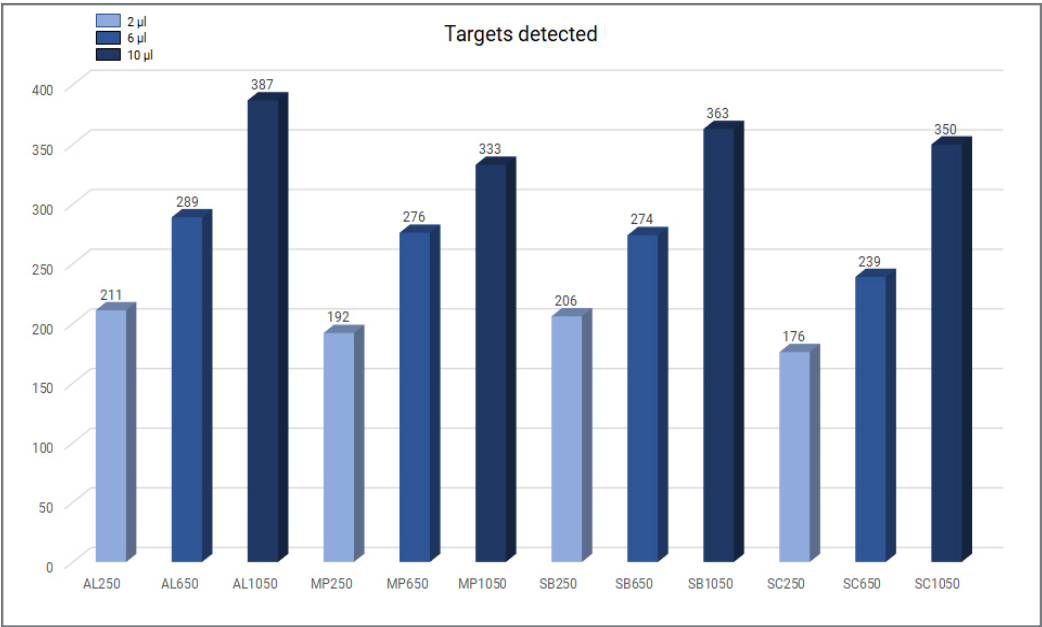


Figure 4. Samples from four healthy donors (AL, MP, SB, SC) were assayed using 3 different input volumes (2 μ l, 6 μ l, 10 μ l) for each sample. The poly-A synthetic oligo was used at 50 amol. Results showed consistency across samples between the volume used and the number of miRNA detected at least by 15 out 20 replicated probe.

Interestingly, more than 70% of expressed miRNAs found in the exosomes were shared across all four healthy individuals. This approach was also implemented in our project profiling exosomal miRNAs derived from plasma samples obtained from 120 subjects with lung cancer. Because of the limited amount of sample available, we chose 6 μ L of purified total RNA as the most suitable volume to be used. We also spiked 50 amol of synthetic oligonucleotide into each hybridization mix to obtain a signal from the Bright Corners. The gridding failed in only one instance, and one sample reported a poor additive error estimation in the green channel. However, repeating the labeling and the hybridization of these samples gave the expected results, indicating that a handling problem had occurred in the first preparation. In addition, six cases showing stains inside their arrays, probably due to washing problems, were replicated reporting excellent QC metrics (Table 1).

	Excellent	Good	Evaluate
IsGoodGrid	127	0	1 ^a
AddErrorEstimateGreen	123	4	1 ^b
AnyColorPrntFeatPopnOL	128	0	0
gNonCtrlMedPrntCVBGSubSignal	122	5	1 ^a

Table 1. Feature Extraction QC Metrics; samples from 120 patients with lung cancer were assayed. One sample (a) did not give good gridding causing the failure of a second metric. For another sample (b) the only AddErrorEstimateGreen metric failed. When the assay was repeated for these two samples (a, b) as well as for other six cases that we decided to replicate, they all gave QC metrics in the excellent range.

Conclusion

When studying exosomal miRNA, or miRNA from very limited input amounts, minimizing the risk of losing precious material is a critical aspect of the experiments. With a couple of modifications, we successfully used the SurePrint human miRNA microarray that allowed us to establish a workflow that avoided the issue of quantifying the input amount, while still giving us satisfactory miRNA measurements for our investigation. Besides, we were able to resolve the occasional failure of the automatic gridding in feature extraction, which had caused the loss of data from limited samples that could not be retested.

We have permanently implemented these modifications in our exosomal miRNA workflow. With this current project studying exosomal miRNA from lung cancer samples on the miRNA microarray, we have obtained data from more than 100 liquid biopsies without losing a single sample.

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